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CONTENTS OF THIS ISSUE

A Kinetic Study of Barbital Degradation in an Ammonia Buffer System 627
J. E. Goyan, Z. I. Shaikh, and *J. Autian*

Heating and Cooling Rate Coefficients and Related Factors Affecting Procedures for Tablet Shelf Life Prediction 632
Stuart P. Eriksen, George M. Irwin, and *Joseph V. Swintosky*

Some Effects of Ionized Air on *Penicillium notatum* 643
Robertson Pratt and *Robert W. Barnard*

Rat Bioassay of Combinations of Diuretics 647
Mario D. G. Aceto and *Casimir T. Ichniowski*

The Determination of Eucalyptol by Residual Titration With Hydrogen Bromide in Acetic Acid 650
Martin I. Blake and *Gilbert Rabjohn*

The Toxicity and Safety Testing of Disposable Medical and Pharmaceutical Materials 652
John H. Brewer and *Harold H. Bryant*

Effects of Tranquilizers on Bacterial Toxemias II. Meprobamate 657
Leo Greenberg and *James W. Ingalls*

Investigation of *Machilus macrantha* Nees. I. Pharmacognostical and Phytochemical Study of Root 659
K. N. Gaind and *S. K. Baveja*

Investigation of *Machilus macrantha* Nees. II. Pharmacological Action and Chemical Constitution of Machiline 663
K. N. Gaind and *S. K. Baveja*

The Selective Determination of Isopropamide Iodide, A Low-Molecular Weight Quaternary Ammonium Compound 666
Ralph S. Santoro

The Biological Action of Cellular Depressants and Stimulants V. The Effect of Phenylurethane on Cellular Synthesis by *Tetrahymena pyriformis* GL 669
Walter Singer and *John J. Eiler*

A Quantitative Fluorometric Reaction for Glutethimide 673
R. P. Haycock, P. B. Sheth, and *W. J. Mader*

Adaptation of the Chromotropic Acid Method to the Assay of Spans 678
Lacey Gatewood, Jr., and *Horace D. Graham*

Note on X-ray Crystallographic Data on Some Alkaloids from *Vinca rosea* Linn 681
Ann Van Camp

Note on the Occurrence of Taxine in *Taxus brevifolia* 683
V. E. Tyler, Jr.

Note on the Disappearance of Pharmacologic Activity 685
Joseph V. Swintosky and *F. M. Sturtevant*

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A Kinetic Study of Barbital Degradation in an Ammonia Buffer System*

By J. E. GOYAN, Z. I. SHAIKH, and J. AUTIAN

The kinetics of the degradation of barbital in an ammonia buffer have been studied. The various species taking part in the parallel reactions leading to decomposition have been taken into account by means of equilibrium constants and, from this, the various second-order rate constants have been calculated. A possible mechanism for the reaction corresponding to known amide hydrolysis mechanisms is postulated.

THE DECOMPOSITION of barbiturates in aqueous solutions has been recognized for many years (1). The various intermediates have been postulated and isolated (1–4). The rate studies, however, have been of a semiquantitative nature and it was felt that a quantitative study of the kinetics of the decomposition was needed.

EXPERIMENTAL

Materials and Apparatus.—Barbital, U. S. P. grade, was recrystallized before use (m. p. 189–190°), all other chemicals were reagent grade. A Beckman model DU spectrophotometer and a Leeds and Northrup model 7664 pH meter were used.

Assay.—The concentration of barbital was followed by determining the absorbance of a 2-cc. sample of the experimental solution, diluted to 100 cc. with ammonia buffer (pH 9.5), at 239 mμ. The spectrophotometric method was chosen since it is rapid and the products of the degradation do not interfere with the determination (2, 5).

Concentration of Barbital as a Function of Time.—Samples of barbital (0.1% w/v final concentration) were made up at four different pH values (approx. pH 8.5, 9.0, 9.5, and 10) using four different concentrations of ammonium chloride for each pH value. The total ionic strength was adjusted to 1.0 with potassium chloride in each case. The samples were sealed in 10-cc. ampuls and kept in a constant temperature water bath at 40, 50, 60, and 65 ± 0.1°.

The reaction followed a pseudo first-order course since the log of the absorbance plotted *vs.* time was a straight line. Table I gives the pseudo first-order rate constants for the different samples, determined by the method of least squares.

DISCUSSION

In order to separate the different factors in the degradation, it is necessary to take into account the different forms which are present in an aqueous solution of a barbiturate. Calculation of the concentrations of the different forms shows that only the unionized and first ionic forms are important at pH values between 8 and 10.[1]

The rate of degradation of the barbiturate, therefore, is dependent on the concentration of these two forms and probably the amounts of acid and base present. This expectation may be formulated as follows: let x = unionized barbiturate, y = ionized barbiturate, z = total barbiturate, H^+ = hydronium ions, OH^- = hydroxide ions, A = acidic form of the buffer, B = basic form of the buffer, $-v$ = rate of disappearance of various forms, k = second-order rate constants for the various possible reaction paths, [ ] denote concentration and ( ) denote activity, f = activity coefficient of the various forms, and K = pseudo first-order rate constant. Then

---

* Received December 7, 1959, from the College of Pharmacy, University of Michigan, Ann Arbor.
Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

[1] By writing out the different equilibria taking place in the solution and using the principle of electrical neutrality, it is possible to get three equations in the three unknown forms of the barbiturate. Solving, one obtains for the fraction in the second ionic form

$$B = \frac{K_1K_2}{K_1K_2 + [H^+]^2 + K_1[H^+]}$$

Using the approximate value of K_2 given by Butler, *et al.* (6), one finds that the fraction is less than 0.01 at pH 10.

TABLE I.—PSEUDO FIRST-ORDER RATE CONSTANTS FOR THE DEGRADATION OF BARBITAL

NH_4^+, M	40° C. pH, 25°	40° C. K, days^{-1}	50° C. pH, 25°	50° C. K, Days^{-1}	60° C. pH, 30°	60° C. K, Days^{-1}	65° C. pH, 30°	65° C. K, Days^{-1}
0.1704	8.60	0.97×10^{-2}	8.60	2.69×10^{-2}	8.50	7.42×10^{-2}	8.60	1.13×10^{-1}
0.3408	8.60	1.01×10^{-2}	8.60	2.81×10^{-2}	8.50	7.84×10^{-2}	8.60	1.14×10^{-1}
0.5112	8.60	1.03×10^{-2}	8.60	3.01×10^{-2}	8.50	8.17×10^{-2}	8.60	1.19×10^{-1}
0.6816	8.60	1.09×10^{-2}	8.60	3.10×10^{-2}	8.50	8.32×10^{-2}	8.60	1.24×10^{-1}
0.1292	9.12	1.20×10^{-2}	9.12	3.38×10^{-2}	9.00	9.60×10^{-2}	9.10	1.45×10^{-1}
0.2584	9.12	1.24×10^{-2}	9.12	3.46×10^{-2}	9.00	9.82×10^{-2}	9.10	1.52×10^{-1}
0.3876	9.12	1.31×10^{-2}	9.12	3.58×10^{-2}	9.00	1.01×10^{-1}	9.10	1.60×10^{-1}
0.5168	9.12	1.36×10^{-2}	9.12	3.77×10^{-2}	9.00	1.04×10^{-1}	9.10	1.61×10^{-1}
0.0731	9.60	1.39×10^{-2}	9.60	3.86×10^{-2}	9.50	1.05×10^{-1}	9.60	1.63×10^{-1}
0.1462	9.60	1.42×10^{-2}	9.60	4.07×10^{-2}	9.50	1.08×10^{-1}	9.60	1.69×10^{-1}
0.2193	9.60	1.50×10^{-2}	9.60	4.35×10^{-2}	9.50	1.13×10^{-1}	9.60	1.77×10^{-1}
0.2924	9.60	1.53×10^{-2}	9.60	4.43×10^{-2}	9.50	1.16×10^{-1}	9.60	1.81×10^{-1}
0.0308	10.10	1.78×10^{-2}	10.10	4.55×10^{-2}	10.00	1.22×10^{-1}	10.10	1.86×10^{-1}
0.0616	10.10	1.82×10^{-2}	10.10	4.67×10^{-2}	10.00	1.24×10^{-1}	10.10	1.91×10^{-1}
0.0924	10.10	1.92×10^{-2}	10.10	4.78×10^{-2}	10.00	1.28×10^{-1}	10.10	1.97×10^{-1}
0.1232	10.10	1.99×10^{-2}	10.10	4.97×10^{-2}	10.00	1.34×10^{-1}	10.10	2.05×10^{-1}

using the usual formulation for general acid-base catalysis (7)

$$-v_x = k_x^{\circ}[x] + k_x^{H^+}[x][H^+] + k_x^{OH^-}[x][OH^-] + k_x^{A}[x][A] + k_x^{B}[x][B] \quad \text{(Eq. 1)}$$

$$-v_y = k_y^{\circ}[y] + k_y^{H^+}[y][H^+] + k_y^{OH^-}[y][OH^-] + k_y^{A}[y][A] + k_y^{B}[y][B] \quad \text{(Eq. 2)}$$

Preliminary experiments indicated that no degradation of barbital took place after fifteen days in 0.1 N hydrochloric acid solution at 60°. From these results it may be assumed that the reaction rate constants k_x° and $k_x^{H^+}$ are negligible in comparison with the other constants and thus may be eliminated. If this is the case, then k_x^{A} may also be disregarded.

Now $[x] + [y] = [z]$
and $x \rightleftharpoons H^+ + y$

$$K_A = \frac{(H^+)(y)}{(x)} = \frac{[H^+][y]}{[z] - [y]} \frac{f_{H^+} f_y}{f_x} \quad \text{(Eq. 3)}$$

since $f_x \cong 1$, and defining $f_{H^+} f_y = f^2$ gives

$$K_A = \frac{[H^+][y]}{[z] - [y]} f^2 \quad \text{(Eq. 4)}$$

solving for y

$$[y] = \frac{K_A[z]}{K_A + [H^+]f^2} \quad \text{(Eq. 5)}$$

similarly

$$[x] = \frac{[H^+]f^2[z]}{K_A + [H^+]f^2} \quad \text{(Eq. 6)}$$

setting; $-v_x - v_y = -v_z$ and letting $\alpha = K_A + [H^+]f^2$, and also noting that $-v_z/[z] = K$, and combining

$$\alpha K = k_x^{OH^-}K_w f^2 + k_x^{B}[H^+][B]f^2 + k_y^{\circ}K_A + k_y^{H^+}K_A[H^+] + k_y^{OH^-}K_A[OH^-] + k_y^{A}K_A[A] + k_y^{B}K_A[B] \quad \text{(Eq. 7)}$$

Further simplification of the above equation may take place if the buffer dissociation is considered

$$K_A' = \frac{(B)(H^+)}{(A)} = \frac{[B][H^+]}{[A]} \frac{f_{H^+}}{f_A} \quad (f_B = 1) \quad \text{(Eq. 8)}$$

define then $K_A^{\circ} = [B][H^+]/[A]$.

Using this information we may rewrite Eq. 7 as follows:

$$\alpha K = k_y^{\circ}K_A + k_x^{OH^-}K_w f^2 + k_y^{H^+}K_A[H^+] + k_y^{OH^-}K_w K_A[1/H^+] + [k_x^{B}K_A^{\circ}f^2 + k_y^{A}K_A + k_y^{B}K_A K_A^{\circ}[1/H^+]][A] \quad \text{(Eq. 9)}$$

This equation shows that a plot of K *vs.* $[A]$ should be a straight line with slope m and y axis intercept of b.

A typical plot is shown for 40° and several pH values, in Fig. 1. In order to interpret the results further it is necessary to find values for the different dissociation constants and activity coefficients.

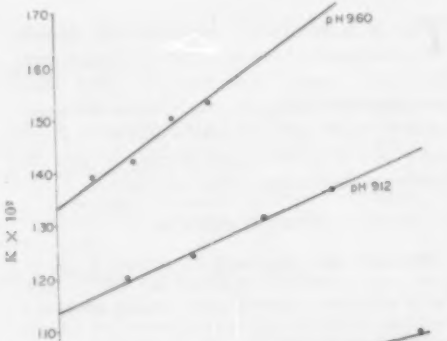

Fig. 1.—Pseudo first-order rate constants plotted *versus* ammonium ion concentration at 40°.

The dissociation constants for the ammonia buffer system were calculated from (8)

$$pK'_A = \frac{2835.76}{T} - 0.632 + 0.001225T \quad \text{(Eq. 10)}$$

the dissociation constants for barbital by (9)

$$pK_A = \frac{2324.47}{T} - 3.349 + 0.01186\,T \quad \text{(Eq. 11)}$$

and (10)

$$pK_w = \frac{4470.99}{T} - 6.088 + 0.01706T \quad \text{(Eq. 12)}$$

The determination of the activity coefficients was a more difficult task. Individual ionic activities are, of course, not defined. However, reasonable values for them can be estimated and used (11). The value for f_B was adopted from the paper by Krahl (12). The activity coefficients for $[NH_4^+]$ and $[H^+]$ were estimated using the MacInnes assumption (13) and data on the mean ionic activities of ammonium chloride, dilute hydrochloric acid, and water given in Harned and Owen (14). The approach using hydrochloric acid gave a value of 0.88 for f_{H^+}. Using water, a value of 0.85 was obtained. We used a value of 0.87 in our work. We assumed a decrease of 0.01 unit to take place between 50 and 60°.

Inspection of Eq. 9 shows that

$$a_m = k_x^B K_A f^2 + k_y^A K_A + k_y^B K_A K_A [1/H^+] \quad \text{(Eq. 13)}$$

and

$$a_b = k_y^o K_A + k_x^{OH^-} K_w f^2 + k_y^{H^+} K_A [H^+] + k_y^{OH^-} K_A K_w [1/H^+] \quad \text{(Eq. 14)}$$

The hydrogen ion concentration was calculated using Eq. 8. It was solved at 25° to obtain C_B knowing the pH. Then with known C_B it was possible to solve for hydrogen ion concentration at the other temperatures, using the appropriate values of K_A' and activity coefficients.

Equation 14 can be written in the form

$$Y = k_1 + k_2[H^+] + k_3[1/H^+] \quad \text{(Eq. 15)}$$

Attempts to fit this curve by the method of least squares gave a negative value for one of the constants. One constant at a time was then dropped and the only reasonable fit to the data was obtained from the equation

$$Y = k_1 + k_2[1/H^+] \quad \text{(Eq. 16)}$$

It was therefore assumed that $k_y^{H^+}$ is equal to zero and consequently k_y^A is also zero. This appears reasonable since $k_x^{H^+}$ and k_x^A are zero. Table II contains the values of k_1 and k_2 calculated by the method of least squares. The values for $k_y^{OH^-}$ are also calculated and given in Table II. Unfortunately, k_1 contains both k_y^o and $k_x^{OH^-}$; however, it is reasonably certain that $k_x^{OH^-}$ is much greater than k_y^o, and k_y^o is therefore neglected. The values for $k_x^{OH^-}$ are calculated and given in Table II.

Equation 13 is of the type (after k_y^A is dropped)

$$Y = k_4 + k_5[1/H^+] \quad \text{(Eq. 17)}$$

The values of k_4 and k_5 were obtained by the method of least squares and the values of k_y^B and k_x^B calculated from these. The values are given in Table III.

Arrhenius type plots of log k *vs.* $1/T$ are shown in Figs. 2–5. The following activation energies are found from these figures: $E_A(k_x^B) = 16.0 \times 10^3$, $E_A(k_y^B) = 16.2 \times 10^3$, $E_A(k_x^{OH^-}) = 14.9 \times 10^3$, and $E_A(k_y^{OH^-}) = 11.4 \times 10^3$, all in calories per mole.

Effect of Ionic Strength.—The effect of ionic strength on a reaction such as this is difficult to predict since there are apparently four different parallel reactions. The final equation may be rewritten in terms of the theory of absolute reaction rates as follows (15):

$$\alpha K = k_x^{OH^-} K_w + \frac{2 f_{OH^-} f_x}{f_{\neq}} + k_x^B [H^+][B] + \frac{2 f_x f_B}{f_{\neq}} + k_y^B K_A [B] \frac{f_y f_B}{f_{\neq}} + k_y^{OH^-} K_A [OH^-] \frac{f_y f_{OH^-}}{f_{\neq}} \quad \text{(Eq. 18)}$$

The activity coefficient ratios for the first three terms may be dropped since the activity coefficient for the activated complex should be approximately equal to the activity coefficient of the numerator.

TABLE II.—*b* ANALYSIS

t, °C.	K_A	K_w	$K_A K_w$	k_1	k_2	f^2
40	1.66×10^{-9}	2.92×10^{-14}	4.85×10^{-23}	1.90×10^{-10}	2.42×10^{-20}	0.41
50	2.04×10^{-9}	5.48×10^{-14}	11.18×10^{-23}	6.88×10^{-10}	1.03×10^{-19}	0.41
60	2.63×10^{-9}	9.61×10^{-14}	25.27×10^{-23}	2.60×10^{-9}	3.79×10^{-19}	0.40
65	2.95×10^{-9}	1.29×10^{-13}	38.08×10^{-23}	4.42×10^{-9}	7.60×10^{-19}	0.40

$$k_y^{OH^-} = \frac{k_2}{K_w K_A}$$

t, °C.	$k_y^{OH^-}$ (L. moles^{-1} days^{-1})	log $k_y^{OH^-}$
40	4.99×10^1	1.70
50	9.23×10^1	1.97
60	1.50×10^2	2.18
65	1.99×10^2	2.30

assuming $k_x^{OH^-} \gg k_y^o$

$$k_x^{OH^-} = \frac{k_1}{K_w f^2}$$

t, °C.	$K_w f^2$	$k_x^{OH^-}$ (L. moles^{-1} days^{-1})	log $k_x^{OH^-}$
40	1.20×10^{-14}	1.58×10^4	4.20
50	2.24×10^{-14}	3.07×10^4	4.49
60	3.84×10^{-14}	6.77×10^4	4.83
65	5.17×10^{-14}	5.17×10^4	4.93

TABLE III.—*m* ANALYSIS

t, °C.	K_A	$K°_A$	k_1	k_2	f^2
40	1.66×10^{-8}	9.73×10^{-10}	2.72×10^{-11}	8.97×10^{-20}	0.41
50	2.04×10^{-8}	1.82×10^{-9}	2.03×10^{-10}	4.00×10^{-19}	0.41
60	2.63×10^{-8}	3.12×10^{-9}	4.37×10^{-10}	2.28×10^{-19}	0.40
65	2.95×10^{-8}	4.26×10^{-9}	13.85×10^{-10}	4.28×10^{-18}	0.40

$$k_2^B = \frac{k_2}{K_A K°_A}$$

t, °C.	$K_A K°_A$	k_2^B (L. moles^{-1} days^{-1})	$-\log k_2^B$
40	1.615×10^{-17}	5.55×10^{-3}	2.26
50	3.713×10^{-17}	1.07×10^{-3}	1.97
60	8.389×10^{-17}	2.72×10^{-3}	1.57
65	12.55×10^{-17}	3.41×10^{-3}	1.47

$$k_1^B = \frac{k_1}{K°_A f^2}$$

t, °C.	$K°_A f^2$	k_1^B (L. moles^{-1} days^{-1})	$-\log k_1^B$
40	3.98×10^{-10}	6.83×10^{-1}	1.17
50	7.47×10^{-10}	2.72×10^{-1}	0.57
60	1.25×10^{-9}	3.49×10^{-1}	0.46
65	1.70×10^{-9}	8.15×10^{-1}	0.09

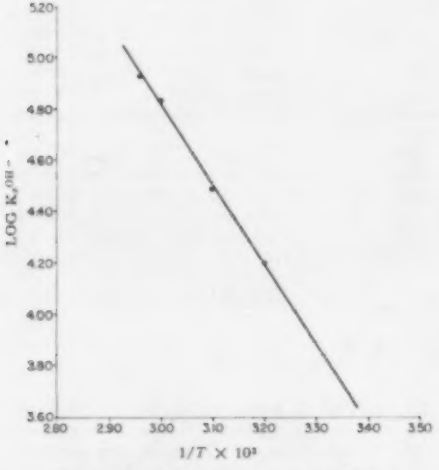

Fig. 2.—Arrhenius type plot for $k_2^{OH^-}$.

The activity coefficient term in α may be ignored as long as K_A is larger than H^+. As a first approximation the activity coefficients may be substituted with the appropriate value from the Debye Hückel limiting law and the derivative of log K with respect to the square root of ionic strength (μ), may be taken. This gives

$$\frac{d \log K}{d\sqrt{\mu}} = \frac{-C_1 10^{-\sqrt{\mu}} + C_2 10^{\sqrt{\mu}}}{C_1 10^{-\sqrt{\mu}} + C_2 10^{\sqrt{\mu}}} \quad \text{(Eq. 19)}$$

Where C_1 and C_2 are the appropriate lumped constants. At a pH of 9.40 and 60°, using the appropriate constants, a value for the derivative of Eq. 19 was found to be a small negative number. Thus, rate of the reaction should fall off slowly with increases in ionic strength under these particular conditions. The values for this particular experiment were determined and are given in Table IV. Table IV shows that the decrease is indeed slow and the equation is at least approximately correct. The effect of ionic strength is thus qualitatively predictable from this approximate equation.

Mechanism.—There are several interesting aspects to this degradation. The apparent lack of acid catalysis is surprising in view of the known acidic hydrolysis of many other amides (16, 17). This may be explainable in terms of the very weak basicity of the imide structure. Attempts to titrate barbital in nitromethane with perchloric acid, according to the method of Streuli (18), failed, indicating that barbital is a weaker base than acetamide. Since protonation is probably an important step in the acidic catalysis of amide hydrolysis (16, 19) this weak basicity of the barbital may account for our inability to show acidic catalysis.

TABLE IV.—THE EFFECT OF IONIC STRENGTH[a]

$\sqrt{\mu}$	$k \times 10^3$, hr.$^{-1}$
0.45	4.45
0.630	4.35
0.775	4.28
0.895	4.23
1.000	4.18

[a] 60° C., pH 9.40.

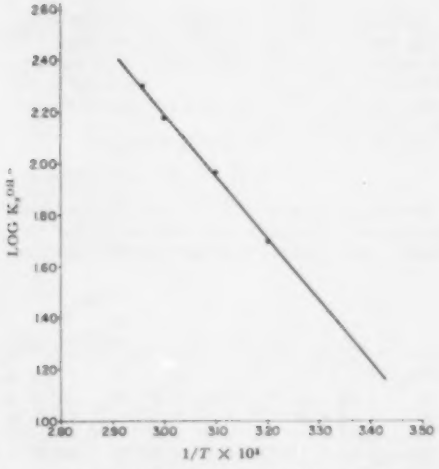

Fig. 3.—Arrhenius type plot for $k_2^{OH^-}$.

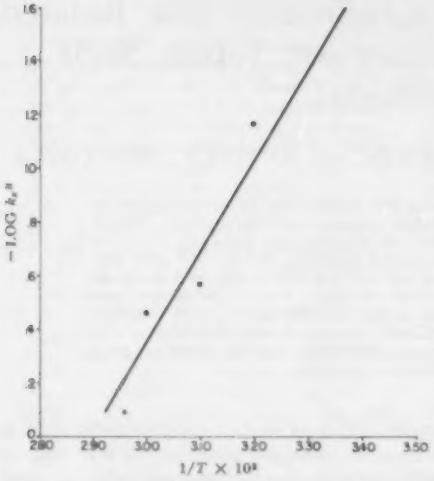

Fig. 4.—Arrhenius type plot for k_a^B.

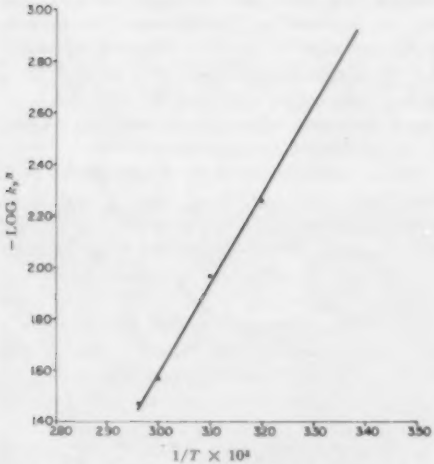

Fig. 5.—Arrhenius type plot for k_b^B.

The basic hydrolysis of the unionized species can be formulated along known pathways as shown in Fig. 6*a*.

The hydrolysis of the ionized species would probably be at the 4 (or 6) position because of the resonance shown in Fig. 6*b*. Hydrolysis at this position would, of course, lead to different products. It is interesting to note in this connection that Fretwurst (4) found that the concentration of diamide (the product of hydrolysis of unionized barbiturate) decreased when the concentration of base was increased. Further work in an attempt to substantiate these mechanisms is in progress.

The catalysis by ammonia is also rather surprising. Perhaps it can be accounted for by visualizing a reaction similar to the ammonolysis of an ester (20).

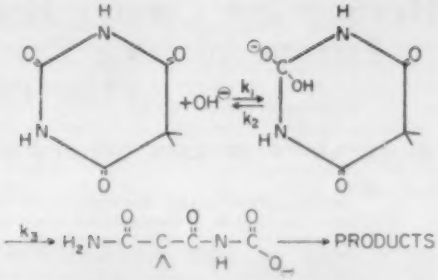

Fig. 6*a*.—Hydrolysis mechanism of unionized barbital.

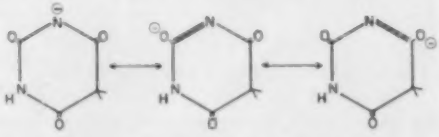

Fig. 6*b*.—Resonance forms of the ion.

The product of the reaction would be quite unstable and a means of verifying this possibility appears rather difficult.

SUMMARY

1. The rate of degradation of barbital has been shown to follow a pseudo first-order reaction.

2. The rate constants for the various parallel reactions which take place have been isolated and evaluated.

3. The possible mechanisms of the various reactions are discussed.

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Heating and Cooling Rate Coefficients and Related Factors Affecting Procedures for Tablet Shelf Life Prediction*

By STUART P. ERIKSEN, GEORGE M. IRWIN, and JOSEPH V. SWINTOSKY

Data are presented for the heating and cooling rate coefficients of tablets under the usual conditions of exaggerated temperature hot air oven stability studies. The interrelationships of tablet composition, container dimensions, the drug activation energy and the probability factor of the Arrhenius equation, the storage oven temperature, time of storage, and sample heating and cooling rate coefficients are noted as they influence the programming and results of short term, high temperature thermal degradation studies. Also discussed is the possible influence of other factors within the control of the development pharmacist which may affect the validity of exaggerated temperature thermal drug degradation data used in room temperature shelf life prediction of products.

THIS PAPER describes a quantitative study of the effects of tablet size, coating, bottle size, position in a bottle, and position in a carton on the heating and cooling characteristics of tablets; also discussed are how the tablet heating and cooling characteristics, storage oven operation and temperatures, and other matters pertaining to the programming of an exaggerated temperature stability study may influence the accuracy of shelf life predictions of drugs in tablets.

An awareness of these factors as they affect the heating and cooling behavior of tablets in commercial containers is important because: (*a*) during the development of new tablet formulations, exaggerated temperature stability studies are usually conducted in containers of size and closure corresponding to those anticipated for the marketed product, (*b*) exaggerated temperature stability studies usually employ rooms or air ovens at temperatures ranging from 30 to 85° for periods as short as several hours to as long as several years, (*c*) the best use of the chemical kinetic approach to shelf life prediction requires accurate knowledge of the time and temperature of exposure of each tablet analyzed, (*d*) analysis of a tableted drug frequently is performed on only a fraction of the tablets in a given bottle or carton, and (*e*) during transit and storage, pharmaceutical products frequently receive short term exposure to temperatures appreciably higher or lower than normal room temperature.

In an earlier report (1) it was suggested that, in exaggerated temperature studies, errors in the estimation of product shelf life might result if no consideration was given to the slow heating and cooling processes characteristic of most pharmaceutical solids. It was proposed that an estimate of exaggerated oven temperature storage time errors involved could be calculated on the basis of an "equilibrium temperature time equivalent" (ETTE). This is an expression for the actual storage time of a product at a given temperature corrected for the effects of the finite times required for heating or cooling to an equilibrium temperature.

In our experimental work thermocouples were introduced into individual tablets, and their temperature histories were recorded as a function of time under conditions similar to those used for stability studies. From these data, the heating and cooling characteristics and the ETTE's were determined for the tablets. Using the ETTE concept, calculations were made showing the magnitude of errors that can occur in studies of drug degradation and product shelf life when oven storage times are not corrected for the effects of the heating and cooling processes. Also, calculations were made of the minimum oven storage times beyond which, under conditions similar to these studies, the storage time errors would be relatively insignificant in the calculation of shelf life from short term exaggerated temperature analytical data.

The study of the degree to which various factors influence tablet heating, cooling, and drug degradation, thus influencing the accuracy of chemical kinetic calculations of drug degradation rates, should be helpful in the programming of accelerated studies of product shelf life. Also, these studies give an indication of the effects that temperature extremes may have on packaged pharmaceutical products during such exposure in transit and storage.

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Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.
The authors gratefully acknowledge the assistance of Mr. J. F. Pauls with the statistical portion of this work.

EXPERIMENTAL

Thermocouples.—Polyvinyl coated, 26 gauge solid-wire, iron-constantan thermocouples were used for all measurements of tablet temperatures. Thermocouple junction wires were twisted, welded with a small torch, and then were trimmed short.

Recording Equipment.—A Minneapolis-Honeywell "Electronik" potentiometric recorder was used to record temperature as a function of time. The span was so adjusted that temperature was recorded directly in degrees centigrade. All measurements were made using a crushed ice-distilled water reference bath at 0°. Under the above conditions the temperature recording was correct to within 0.5% over a temperature span of 0 to 100°.

Oven.—A large (27 cu. ft.), mechanically convected hot-air oven (Hotpack model 1204) was used for all storage studies. The temperature regulation of this oven showed a variation of less than ±2°.

Tablets.—Three different tablets were used: (*a*) Large pink sugar-coated convex vitamin tablets; 1/2 inch (1.27 cm.) diameter, 7/32 inch (0.56 cm.) thick, and 0.80 Gm. weight. (*b*) Small gray sugar-coated convex calcium sulfate tablets; 3/8 inch (0.95 cm.) diameter, 7/32 inch (0.56 cm.) thick, and 0.43 Gm. weight. (*c*) Small uncoated convex aspirin tablets; 5/16 inch (0.79 cm.) diameter, 7/32 inch (0.56 cm.) thick, and 0.30 Gm. weight.

The thermocouples were inserted into the tablets through holes (No. 60) drilled radially, two-thirds the distance through the tablet. The thermocouples were sealed in the holes with a droplet of deKhotinsky cement (Fig. 1). Several thermocouples were inserted into uncoated tablets during the punching process, using a slotted die (Fig. 2).

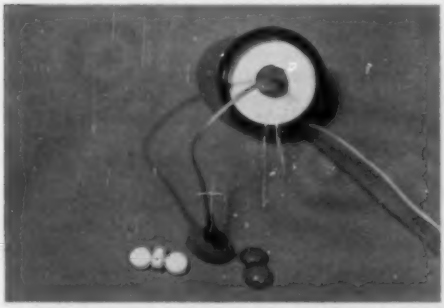

Fig. 1.—Picture showing drilled tablets and placement of thermocouple.

Containers.—Standard round amber-glass powder bottles (20,[1] 28,[1] 50, 60, 100, 120, 200, and 250 ml.) with plastic polyvinyl-cardboard lined screw caps were used to hold tablet samples. A one-fourth-inch hole was drilled through the center of the cap and liner to allow the thermocouple wire to be led into the tightly capped bottle. The hole was sealed and the wire and tablet held in place with a cork stopper (Fig. 3). The tablet with the inserted thermocouple was placed in a desired position within the bottle. Then the remaining tablets were poured in and the cap was screwed tight.

[1] These were commercial 5- and 7-dr. vials.

Standard pasteboard cartons holding 12 bottles each were used for the carton tests. In each case, all 12 bottles contained the same type of tablets as those being measured, and the tablet being studied was located centrally in the bottle. The arrangement of the bottles in the carton is shown in Fig. 4. Reference to Fig. 4 clarifies the designations: corner of carton (COC), side of carton (SOC), and interior of carton (IOC). The temperature of each of the three tablets in the three respective bottles was

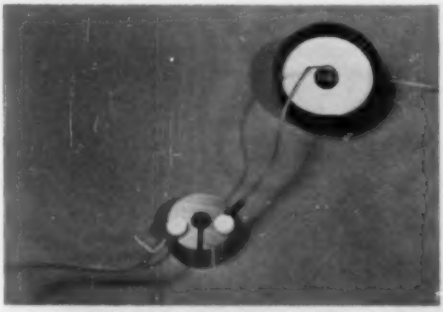

Fig. 2.—Picture showing thermocouple attached to tablet by use of a slotted die during compression.

Fig. 3.—Picture showing the drilled cap, stopper holding the thermocouple wire, and tablet placement in the bottle.

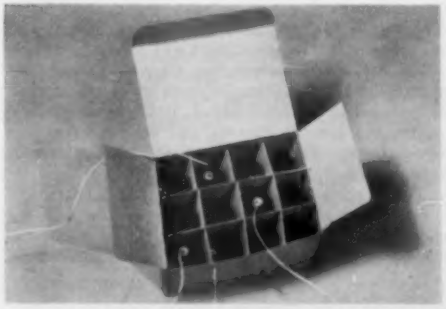

Fig. 4.—Picture showing the arrangement of bottles in cartons where thermocouples sealed in tablets were situated in the center of respective bottles.

checked and recorded every two minutes with the aid of an automatic switching device (Fig. 5). This device was assembled from a 30 r. p. h. synchronous motor, four adjustable cams, and four microswitches.

In studies on helium-filled containers, bottles filled with tablets were inverted and flushed with helium gas (Matheson Co.). The caps were tightened, and the heating and cooling curves were determined with the bottles inverted.

Temperature Measurement Program.—Heating and cooling measurements of temperature *versus* time at three oven temperatures, 45, 60, and 75°, were made on tablets located both centrally and laterally (against the glass) in eight different-sized bottles, and in three bottle positions within the cartons.

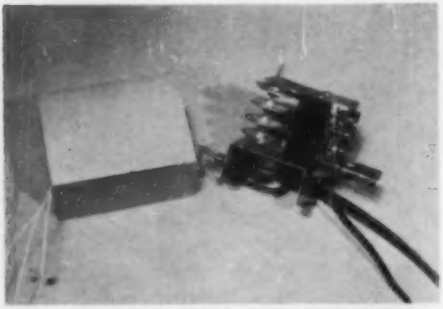

Fig. 5.—Picture showing automatic switching device for monitoring temperatures of tablets within bottles contained in closed pasteboard cartons.

THEORY AND CALCULATIONS

Ball and others (2, 3, 4) have reported that canned foods heated in ovens or retorts tend to heat over the greater portion of their heating range according to a process which may be expressed as

$$\log(T_{ov} - T) = \frac{-Z_h}{2.303} t + \log(T_{ov} - T_r) \quad \text{(Eq. 1)}$$

Cooling may be represented by the equation[1]

$$\log(T - T_r) = \frac{-Z_c}{2.303} t + \log(T_{ov} - T_r) \quad \text{(Eq. 2)}$$

The terms $Z_h/2.303$ and $Z_c/2.303$ are determined experimentally by plotting $\log(T_{ov} - T)$ or $\log(T - T_r)$, respectively, *versus* time. The slopes of the straight line portions of such plots are numerically equal to $Z_h/2.303$ and $Z_c/2.303$, where Z_h and Z_c are the specific heating and cooling rates (1), respectively. Figure 6 is an illustration of this type of plot.

The heating and cooling rates of the contents of a bottle are related to bottle dimensions and the thermal conductivity, specific heat, and density of the bottle and its contents (2) by the expression

$$Z = \frac{4K}{F_\pi} \times \frac{1}{D^2} \quad \text{(Eq. 3)}$$

[1] Definitions of terms are included in the text only where needed for clarity. A complete glossary is appended.

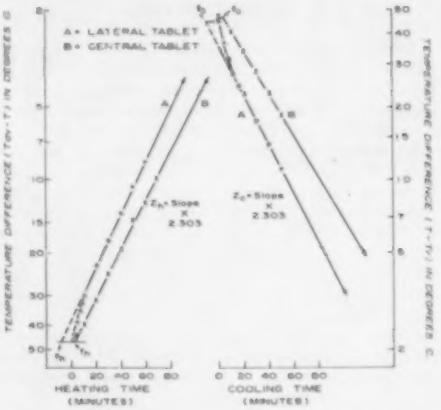
A = LATERAL TABLET
B = CENTRAL TABLET
TEMPERATURE DIFFERENCE (To - T) IN DEGREES C.
TEMPERATURE DIFFERENCE (T - Tr) IN DEGREES C.
Zh = Slope x 2.303
Zc = Slope x 2.303
HEATING TIME (MINUTES)
COOLING TIME (MINUTES)

Fig. 6.—Graph showing the relationship of factors f_h, f_c, Z_h, and Z_c in heating and cooling plots for large coated tablets in 250-ml. bottles. Room and oven temperatures were 27 and 75°, respectively. The data obtained from these plots are:

	Z_h	f_h	Z_c	f_c
Central	0.023	3	0.020	4
Lateral	0.024	−9	0.023	−9.5

where Z is either Z_h or Z_c, and K is the diffusivity constant which may be defined by

$$K(\text{Diffusivity}) = \frac{\text{Thermal Conductivity}}{\text{Density} \times \text{Specific heat}} \quad \text{(Eq. 4)}$$

Each term on the right of the equality sign in Eq. 4 refers to constants which are characteristics of the bottle plus its contents. The term F_π is a dimensional constant controlled by the ratio of bottle diameter, D, to height, H, in the expression

$$\frac{1}{F_\pi} = \frac{1}{2.303}\left[5.783 - 2.467\left(\frac{D}{H}\right)^2\right] \quad \text{(Eq. 5)}$$

This equation is obtained algebraically from the general expression of Williamson and Adams (5) for the sudden heating or cooling of a cylindrical solid.

Plots of Z *versus* $1/D^2$ should yield straight lines with a slope of $4K/F_\pi$ for bottles and containers all having identical diffusivities and the same ratio of D/H (2). If, as in our studies, dimensions change while the density of the contents remains essentially constant, plots of $1/D^2F_\pi$ (essentially dimensional characteristics of the container) *versus* Z should yield straight lines of slope $4K$. The term K is a thermal characteristic of the bottle plus contents.

To calculate the relative storage time errors resulting from the assumption that all tablets in the container are exposed to temperatures and times of storage corresponding to actual oven temperature and the actual period of oven storage, ETTE's for tablets at the sides and centers of various bottles and cartons have been calculated. The derivation and discussion of these equations have been presented previously (1). The per cent storage time error may be calculated as

$$\text{per cent storage time error} = 100 \times \frac{t_2 - \text{ETTE}}{\text{ETTE}} \quad \text{(Eq. 6)}$$

where ETTE has been defined as

$$\text{ETTE} = t_2 - t_1 + t_h + (t_c - t_2) \quad \text{(Eq. 7)}$$

and t_h and $(t_c - t_2)$ as

$$t_h = \frac{A_m \int^{t_1} e^{-\Delta H/R(T_{ov} - (T_{ov} - T_r))\theta - Z_h t}\, dt}{k_{ov}} \quad \text{(Eq. 8)}$$

$$(t_c - t_2) = \frac{A_m \int^{t_2} e^{-\Delta H/R(T_r + (T_{ov} - T_r))\theta - Z_c t}\, dt}{k_{ov}} \quad \text{(Eq. 9)}$$

The terms t_0, t_1, t_2, t_3 are the time of placement in the oven, the time when the sample reaches oven temperature, the time of removal from the oven, and the time when the sample reaches room temperature again, respectively. Simplified solutions to Eqs. 8 and 9 are presented in our previous paper (1).[1]

If a tablet that is under observation does not commence heating or cooling instantly, the time required to begin heating or cooling should be included in the expression for ETTE (Eq. 7). To simplify the calculation of these times, we have idealized the heating and cooling curves at the early time periods, and defined the time required to begin heating or cooling according to these simplified curves. As we define it, the time required for a sample to begin an exponential rate of heating or cooling is determined graphically by extrapolating the straight line portion of the logarithmic plot back to the horizontal line which corresponds to the starting temperature difference, log $(T_{ov} - T_r)$. The f_h and f_c values may be read directly from the intersection of the appropriate log $(T_{ov} - T_r)$ line and the extrapolated lines. This graphical determination of f_h and f_c is illustrated in Fig. 6. If the initial tablet heating or cooling rate is relatively fast, the intersection of the lines may occur somewhat before the actual starting time. When the initial rate is relatively slow, the intersection may occur after starting time.

If cognizance is taken of this initial departure from exponential heating and cooling, Eq. 7 becomes

$$\text{ETTE} = t_2 - t_1 + f_c + t_h + (t_c - t_2) \quad \text{(Eq. 10)}$$

The term t_1 is the difference between the time when the sample is placed in the oven and the time when it reaches oven temperature, so that it includes the factor f_h. Theoretically, t_1 would be infinite, due to the logarithmic relationship between the temperature and time. For purposes of calculation we can overcome this problem, introducing only a minor error, by letting the time when the sample reaches oven temperature be equal to the time when the sample reaches a temperature at which the drug degradation rate is 95% of the value it would have at the equilibrium oven temperature (1). The temperature at which this degradation rate is reached can be calculated readily from the kinetic relationship

$$T_{95\%} = \frac{\Delta H/2.303R}{\Delta H/2.303R \times (1/T_{ov}) - \log 0.95} \quad \text{(Eq. 11)}$$

The time to reach this temperature (t_1) is found from the equation

$$t_1 = 1/Z_h(2.303[\log(T_{ov} - T_r) - \log(T_{ov} - T_{95\%})]) + f_h \quad \text{(Eq. 12)}$$

or

$$t_1 = \frac{Q}{Z_h} + f_h \quad \text{(Eq. 12)}$$

Combining Eqs. 10 and 12, the value of ETTE is

$$\text{ETTE} = t_2 + f_c \frac{-Q}{Z_h} - f_h + t_h + (t_c - t_2) \quad \text{(Eq. 13)}$$

RESULTS AND DISCUSSION

Dimensions and related information regarding tablets, bottles, and cartons used in this study are given in Table I.

It was noted early in our studies that thermocouples sealed into drilled holes gave data identical with those inserted during compression, so this distinction is not made in the data reported.

Specific Rates of Heating and Cooling.—In the heating and cooling experiments, the recordings of the Minneapolis-Honeywell recorder were obtained in degrees centigrade, *versus* time in minutes. These data were replotted according to Eqs. 1 and 2 as log $(T_{ov} - T)$ or log $(T - T_r)$ *versus* time. The slopes of the straight line portions of these plots were equal to $Z/2.303$ from which the values of Z_h and Z_c are readily determined. Extrapolations of the straight line plots of these data also permit determinations of the intercept values of f_c and f_h. Illustrative data for large coated tablets in a 250-ml. bottle at an oven temperature of 75° and a room temperature of about 25° are given in Fig. 6. In this illustration, a tablet located at the side of the bottle shows a Z_h and Z_c of 0.024 and 0.023 min.$^{-1}$, respectively. Values for f_h and f_c are −9 and −9.5 minutes, respectively. A tablet located at the center of the bottle gives values of Z_h = 0.023 min.$^{-1}$, Z_c = 0.020 min.$^{-1}$, f_h = 3 minutes, and f_c = 4 minutes.

It is of interest that values of Z_h for side or centrally located tablets are virtually identical for any given bottle. A similar relationship exists for values of Z_c. Values of f_h for side and centrally located tablets are different, and may have opposite signs, as illustrated above. Similarly f_c values are different under these conditions. Specific heating and cooling rate data for all bottles indicated that Z values increased as bottle size decreased. Also, the Z_h for a given type tablet and bottle was always larger than the Z_c.

[1] Numerical solutions of the integrals of Eqs. 8 and 9 were presented in the previous work in the form,

$$t_h = P_h/Z_h = \frac{P_h}{2.303 \times \text{heating slope}} \quad (a)$$

$$(t_c - t_2) = P_c/Z_c = \frac{P_c}{2.303 \times \text{cooling slope}} \quad (b)$$

where values for P, for a room temperature of 25°, were given as

Temperature, °C.	P_h	P_c
45	2.308	1.241
60	2.328	0.7568
75	2.423	0.5080

In our previous paper (1) Eq. 27 on page 699 is in error. The correct equation is shown above as (*b*).

TABLE I.—PHYSICAL DATA FOR VARIOUS BOTTLES AND CARTONS USED IN TABLET HEATING AND COOLING STUDIES

Bottles

Bottle, ml.	Height, cm.[a]	Diameter, cm.	F_π	Tablets per Bottle[b]		
				Large Coated	Small Coated	Small Uncoated
20 (5 dr.)	4.8	2.6	0.418	18	44	40
28 (7 dr.)	5.3	3.2	0.284	24	57	53
50	5.5	4.2	0.177	51	129	118
60	5.8	4.4	0.161	68	170	157
100	6.8	4.8	0.132	110	270	260
120	7.5	5.4	0.105	135	345	320
200	9.0	6.0	0.083	225	565	530
250	10.0	6.4	0.072	280	705	670

Cartons

Contents	Length, cm.	Width, cm.	Height, cm.
Twelve 28-ml. (7-dr. vials)	13.2	9.9	7.1
Twelve 50-ml. bottles	17.4	12.8	7.8

[a] The height is the distance from the bottom of the bottle to the neck, excluding the cap and thread.
[b] In all instances bottles were filled to the neck with tablets.

Since Z_c and Z_h were determined experimentally, and D and F_π are bottle dimensional constants having the values given in Table I, it is possible to utilize Eqs. 3 and 4 to obtain plots of Z *versus* $1/D^2F_\pi$. Such plots are illustrated in Figs. 7 and 8. The plots show a close approach to linearity in all heating and cooling studies utilizing containers of 28 ml. or larger volumes. The Z values for the 20-ml. bottle show considerable deviation from the straight line toward a lower slope. The linearity of the data for bottles in excess of 28 ml. indicates a fairly uniform value for thermal conductivity (K of Eq. 3) in these bottled tablets. The lower diffusivity observed for the 20-ml. bottle of tablets suggests that in such small containers, heat transfer has become more dependent upon conduction of the tablet material alone rather than on the tablet-air matrix, as appears to be the case with the larger bottles.

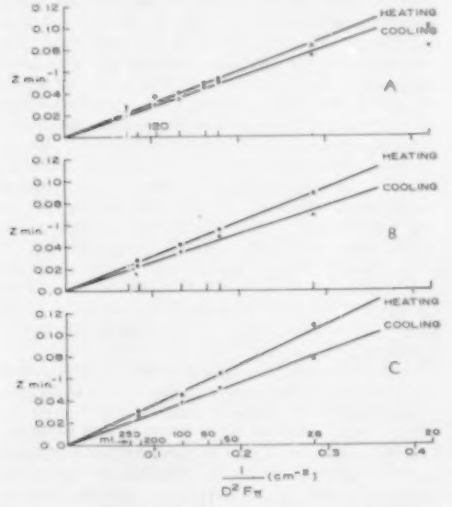

Fig. 7.—Graphs showing the relationship of Z_h and Z_c to $1/D^2F_\pi$ for three tablets, centrally located. *A*, large coated tablets; *B*, small coated tablets; *C*, small uncoated tablets.

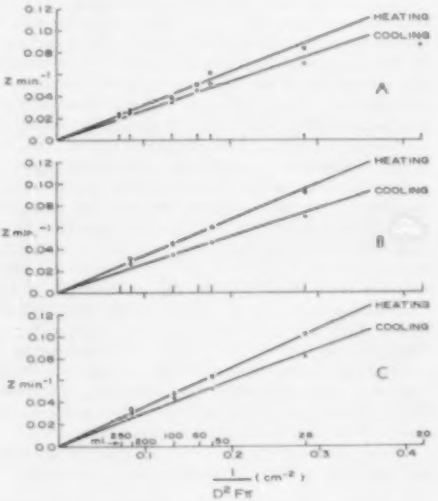

Fig. 8.—Graphs showing the relationship of Z_h and Z_c to $1/D^2F_\pi$ for three tablets, laterally located. *A*, large coated tablets; *B*, small coated tablets; *C*, small uncoated tablets.

Excluding the data for the 20-ml. bottles, the slope of the best straight line, determined mathematically, using the method of least squares, was obtained for plots of Z *versus* $1/D^2F_\pi$ for each heating and cooling experiment performed in these studies. Pooling all of the slopes of these plots in various ways, i. e., all heating slopes *versus* all cooling slopes, all large coated tablets *versus* all small coated tablets, etc., an analysis of variance was made to determine the significance of the observed differences of slope resulting from four of the factors with which we were concerned. These factors were (*a*) heating as compared to cooling, (*b*) central tablets as compared to lateral tablets, (*c*) coated as compared to uncoated tablets, and (*d*) large coated as compared to small coated tablets. Results of this analysis of variance, given in Table II, indicate that only the slopes of

(*a*) heating as compared to cooling, and (*b*) coated as compared to uncoated tablets, were significantly different.

On the basis of these results, all the Z *versus* $1/D^2F_w$ slopes were divided into four groups, i. e., those derived from (*a*) heating of coated tablets, (*b*) heating of uncoated tablets, (*c*) cooling of coated tablets, and (*d*) cooling of uncoated tablets. The slope of the best line fitting each of these groups was calculated, along with the standard error, and is given in Table II. Since the standard errors are small, the average values of these slopes in Table II may be used to write specific equations to describe the Z_h and Z_c values one would obtain for these tablets under these stability study conditions for cylindrical bottles ranging in size from 28 to 250 ml. Substituting these average slope values for $4K$ in Eq. 3, the following equations may be obtained:

$$Z_h(\text{coated tablet}) = 0.3114(1/D^2F_w) \quad \text{(Eq. 14)}$$

$$Z_c(\text{coated tablet}) = 0.2608(1/D^2F_w) \quad \text{(Eq. 15)}$$

$$Z_h(\text{uncoated tablet}) = 0.3628(1/D^2F_w) \quad \text{(Eq. 16)}$$

$$Z_c(\text{uncoated tablet}) = 0.2913(1/D^2F_w) \quad \text{(Eq. 17)}$$

These equations were used to assign average Z values to the 28- to 250-ml. bottles employed in this study. These values are recorded in Table IIA. It should be noted that these Z values are independent of the oven temperatures.

The results summarized in Tables II and IIA indicate that (*a*) the specific heating and cooling rates (Z values) for a given type tablet and bottle under these stability study conditions differ by about 20%. The Z values for heating are always greater. (*b*) Within the tablet size range studied, specific heating and cooling rates are not influenced significantly by tablet dimensions. (*c*) The specific heating and cooling rates of tablets are the same for those located at the center or side of the bottle. (*d*) The uncoated tablets heat and cool significantly faster than similar coated ones. These Z values differ by about 15%. (*e*) Specific heating and cooling rate coefficients are related directly to the thermal conductance and inversely to the product of density, specific heat, cylindrical bottle diameter squared, and the dimensional constant, F_w, for each bottle of tablets considered.

Equations 1–4 and 14–17 do not suggest why specific heating and cooling rates should differ significantly, since thermal conductance and diffusivity should be the same for both heating and cooling. These significant differences are probably due to the conditions under which the heating and cooling studies were performed. In conformance with the usual procedure for performing exaggerated temperature stability studies, during the oven heating stage the bottled tablets are exposed to a rapidly moving air atmosphere which is actuated by a blower fan; however, during the cooling phase, bottled tablets are removed from the oven and allowed to stand in relatively still room air. It is very likely that the specific heating and cooling rates of a given bottle of tablets would become identical if, after heating in a blower oven, they were transferred to an identical blower oven with air being blown at room temperature.

TABLE II.—SLOPE VALUES OBTAINED FROM Z *Versus* $1/D^2F_w$ PLOTS

Division of Data	Slope	Results of Analysis of Variance on Observed Slope Differences
All heating data[a] *versus* All cooling data	0.3295 0.2729	Significant at $P = 0.01$
All central data[a] *versus* All lateral data	0.2995 0.3043	Not significant
All coated tablet data[a] *versus* All uncoated tablet data	0.2868 0.3267	Significant at $P = 0.01$
All large coated tablet data[a] *versus* All small coated tablet data	0.2835 0.2908	Not significant
Coated-heating data	0.3114 ± 0.0052[b]	
Coated-cooling data	0.2608 ± 0.0053	
Uncoated-heating data	0.3628 ± 0.0070	.....
Uncoated-cooling data	0.2913 ± 0.0069	

[a] As explained in the text, the data were divided into two groups, the slope of each group calculated, and an analysis of variance made to test the significance of the difference between the slopes. There were four criteria for making the initial division and each was tested.

[b] After determining the significant differences, the data were divided into four groups, each already proved different from the other, and the slopes and standard errors were calculated.

TABLE IIA.—THE MEAN EXPERIMENTAL Z VALUES (SPECIFIC HEATING AND COOLING RATES) FOR BOTTLES OF TABLETS

Container, ml.	Coated Tablets[a]		Uncoated Tablets[a]	
	Z_h, min.$^{-1}$	Z_c, min.$^{-1}$	Z_h, min.$^{-1}$	Z_c, min.$^{-1}$
28 (7 dr.)	0.088 ± 0.002	0.074 ± 0.002	0.103 ± 0.002	0.083 ± 0.002
50	0.055 ± 0.001	0.046 ± 0.001	0.064 ± 0.001	0.052 ± 0.001
60	0.050 ± 0.001	0.042 ± 0.001	0.058 ± 0.001	0.047 ± 0.001
100	0.041 ± 0.001	0.034 ± 0.001	0.048 ± 0.001	0.039 ± 0.001
120	0.033 ± 0.001	0.027 ± 0.001	0.038 ± 0.001	0.031 ± 0.001
200	0.026 ± 0.0004	0.022 ± 0.0004	0.030 ± 0.001	0.024 ± 0.001
250	0.022 ± 0.0004	0.019 ± 0.0004	0.026 ± 0.001	0.021 ± 0.001

[a] These results are calculated with Eqs. 14–17. Values of D and F_w, required for the calculations, are obtained from Table I.

The significantly higher specific rates of heating and cooling of uncoated tablets, as contrasted with coated tablets, is probably due to an actual difference in thermal diffusivity. It is possible that the white uncoated tablets aid radiant heat transfer through the bottle. Heat transfer mechanism is discussed further in a following paragraph.

Table III gives specific heating and cooling rate data for bottles of tablets in cartons. The same heating and cooling processes appear to be followed whether bottles are in or out of cartons. It was noted, however, that the time required to approach an exponential rate of heating and cooling, f_h and f_c, was longer in cartons. Also values of Z_h and Z_c for bottles in cartons are appreciably smaller than for the same bottles outside of cartons. Position of bottles in cartons also has a bearing on the magnitudes of Z_h and Z_c. These magnitudes bear the following interrelationship COC > SOC > IOC. In the two carton sizes studied, Z_h and Z_c values were smaller in the larger carton. This result was expected and is similar to results between different sized bottles alone. No further studies were done with bottled tablets in cartons.

TABLE III.—TABULATED VALUES OF Z_h AND Z_c FOR TABLETS IN VARIOUS BOTTLES AND CARTONS

Container	Sample Position	Z_h	Z_c
50-ml. bottle	Center	0.055	0.046
100-ml. bottle	Center	0.041	0.034
Carton containing 28-ml. bottles[a]	Center, interior of carton	0.016	0.017
	Center, side of carton	0.019	0.020
	Center, corner of carton	0.025	0.026
Carton containing 50-ml. bottles[a]	Center, interior of carton	0.012	0.011
	Center, side of carton	0.015	0.013
	Center, corner of carton	0.019	0.017

[a] In these cases uncoated tablets were used. The values for 50- and 100-ml. bottles were obtained with coated tablets.

Thermal Diffusivity.—The approximate linearity of Z *versus* $1/D^2F_w$ plots permit an estimate of thermal diffusivity to be made. According to Eq. 3, the slope of the straight line portion of these curves equals $4K$. Values for K have been calculated from the slope values given in Table II and are given in Table IV. Literature values of K for the various materials constituting a bottle of tablets are also included in Table IV. By comparing these data, an estimate may be made of the contribution of the three basic types of heat transfer that are most probable here: solid-solid conduction, gas-solid conduction, and radiation.

The diffusivity of bottled tablets is much too high for heat transfer to be largely dependent upon solid-solid conduction. The increase in K caused by replacing air in bottles with helium is very small compared to the difference between the K values of these two gases, and suggests that gas-solid conduction contributes very little to total heat transfer. The major heat transfer thus appears to occur through a radiation mechanism. Convection undoubtedly plays some part in the heat transfer; however, the tablets offer such a considerable obstacle to gas circulation in the bottle that its contribution to lateral heat transfer must be very small.

TABLE IV.—VALUES OF THERMAL DIFFUSIVITY FOR TABLET FILLED BOTTLES AND TABLET AND BOTTLE MATERIALS

	K, cm.²/sec.
Coated tablets in bottles	
Heating	0.078 (0.082)[a]
Cooling	0.065
Uncoated tablets in bottles	
Heating	0.091
Cooling	0.073
Calcium sulfate ($CaSO_4 \cdot 2H_2O$)	0.003[b]
Sugar	0.005[b]
Glass	0.003[b]
Air	0.2[b]
Helium	2.0[b]

[a] The figure in parenthesis is the K for a helium-filled bottle full of tablets. The other tablet values are for air-filled bottles. These values are calculated from the slope of the best line for the plot of $Z = 4K(1/D^2F_w)$.

[b] These values are estimates of diffusivity for the various components of bottled tablets. They were calculated from the equation K = (thermal conductance/density ×·specific heat). The values for thermal conductance, density, and specific heat were obtained from the "Handbook of Chemistry and Physics," 32nd edition.

Time for Beginning of Exponential Heating and Cooling.—As has been noted, extrapolations of the straight line portions of data relating log $(T_\infty - T)$ or log $(T - T_\infty)$ with time, permit estimates of f_h and f_c. The latter terms were found to range in magnitude from −10 to +15 minutes, with a mean of +3.3 minutes. For tablets located in the central portions of bottled tablets f_h and f_c values increase with increasing bottle size, while for tablets located at the sides of bottles, the f values have small positive values, or have negative values, indicating that the tablets begin to heat or cool much more rapidly than the centrally located tablets.

From Eq. 13 it can be seen that the difference, $f_h - f_c$, rather than the absolute magnitudes of either f_h or f_c is important in the calculation of the ETTE value.

Visual inspection of the data for any single bottle indicates that the f_h and f_c values for any single tablet of a given bottle were not appreciably different. Statistical evaluation of these data indicated that the mean difference for $f_c - f_h$ is only +0.56 minutes, with a standard deviation of 1.64. The difference between f_c and f_h for any single bottle is so small with respect to the usual ETTE value, in a practical stability study, that the f terms may be omitted from Eq. 13 without introducing significant error.

Tablet Temperature Fluctuations.—One would expect that f, Z, K, oven cycle time, and oven temperature variations from the average would be interrelated factors influencing tablet temperature fluctuations during oven storage. For tablets in different positions, bottles, and cartons, where f_h or f_c are less than the oven heating cycle, the temperature of a tablet may fluctuate with the same frequency as the oven cycle, but with less variation; however, when the times f_h or f_c are more than the oven cycle time, tablet temperature fluctuations would tend to become extremely small. In our studies the oven heating cycle time was 3–7 minutes, with a variation from average of ±1 to 1.5°.

Under these heating conditions, no measurable tablet temperature fluctuations were detected. Therefore the study of factors that might influence tablet temperature fluctuations was not pursued further.

Factors Governing the Effective Tablet Storage Times at Exaggerated Temperatures.—The errors in kinetic calculations of drug degradation and predictions of shelf life that result from assuming that actual oven storage time and effective storage time are identical are governed by three principal factors: (*a*) the position of the tablet in the bottle or carton, (*b*) the approximately exponential heating and cooling processes along with duration of oven storage, (*c*) the kinetic constants of the breakdown reaction.

It has been noted already that the effects of f_h and f_c, for any given tablet, may be assumed to cancel each other under conditions of our studies. Therefore, the fact that values may vary from one tablet to another in a given bottle does not introduce error in the determination of storage time under practical exaggerated stability study conditions.

Since the ETTE is independent of f_h and f_c, and Z_h and Z_c are independent of tablet position, there should be little or no differences in total thermal drug degradation from one tablet to another resulting from differences in tablet position within any single bottle. Likewise, there should be no differences in total thermal drug degradation between tablets in different bottles of similar dimensions which are not in cartons. These deductions are based upon the assumption that the storage oven is operating satisfactorily, is not overloaded, and has a relatively uniform and unobstructed flow of the hot air past each bottle. The previous discussion suggests that if bottle size and fill are kept uniform and the foregoing assumptions met, analytical results of tablet drug content following oven storage should not show significant variation from bottle to bottle nor tablet to tablet within a bottle. We have noted that Z_h and Z_c are different for tablets in bottles having different positions within cartons. Taking the IOC position as the reference standard, the error in storage time at other bottle positions, when no correction for position is made, may be calculated from the equation

$$\text{per cent error} = 100 \times \frac{\text{ETTE (IOC)} - \text{ETTE (Other Position)}}{\text{ETTE (IOC)}} \quad \text{(Eq. 18)}$$

Using Eq. 18, positional storage time errors have been calculated for two different sized cartons for three energies of activation. These are given in Table V from which it may be seen that positional storage time errors increase with carton size. From a practical standpoint, analytical protocols and interpretations of analytical data from exaggerated temperature stability studies would be more straightforward if the bottles were not enclosed in cartons. Therefore it is suggested that such studies never utilize bottles in cartons.

Effective oven storage times are influenced appreciably by the fact that heating and cooling are approximately exponential processes. The magnitude of influence is related to the bottle sizes and actual oven storage times employed. The larger the bottles, the longer the periods for attaining equilibrium oven or room temperatures. The longer the storage times, the less will be errors resulting from the assumption that the actual storage time is equivalent to the effective storage time at a given temperature. The error that results from the latter assumption may be calculated from Eq. 6

$$\text{per cent storage time error} = 100 \times \frac{t_s - \text{ETTE}}{\text{ETTE}} \quad \text{(Eq. 6)}$$

where t_s is the actual oven storage time.

One may calculate the effect on the ETTE of the three independent variables, $t_{o'}$, ΔH, and A, by utilizing Eqs. 8, 9, and 10. If actual oven time is constant and is of sufficient duration to enable the sample to reach oven temperature, calculations lead to the following conclusions: (*a*) When $T_{o'}$ is the only variable, there is an increase in the heating time equivalent, t_h, and a decrease in the cooling time equivalent, $(t_s - t_h)$ as $T_{o'}$ increases. The ETTE decreases as the difference between $T_{o'}$ and T_r increases. (*b*) When ΔH is the only variable, t_h increases and t_c decreases as ΔH increases. The net result is that the ETTE decreases with increasing ΔH. (*c*) The kinetic factor, A, has no effect on the magnitude of the ETTE, but it has a marked effect upon the magnitude of the specific rate constant for the degradation reaction.

The calculated ETTE values and per cent errors for uncorrected storage times in a hypothetical stability study are given in Table V, for ΔH values of 15, 30, and 45 Kcal. Under the experimental conditions of our studies, the errors for uncorrected storage times during, for example, a six-hour period, range from 6–37% for a ΔH of 15 Kcal. to 15–157% for a ΔH of 45 Kcal. It may be noted that the importance of the storage time error in practical exaggerated temperature stability studies is relatable to the magnitude of the probability factor A. For those drugs which possess kinetic constants where, relatively speaking, A is small and ΔH is large, thermal stability will be exceptionally great. Six hours of storage at an exaggerated temperature may result in the thermal degradation of only a fraction of 1% of total drug. Under such conditions a storage time error of ±2 hours, for example, would have no important bearing on the determination of the specific velocity constant for thermal degradation, k, in prediction of shelf life. In such an instance relatively long oven storage times would be required to effect appreciable drug degradation at the elevated temperature. Under the conditions of our experimental studies this would signify that the ETTE values in a kinetic study would approach actual oven storage times, whereupon it becomes unnecessary to make storage time corrections.

Under conditions where A is relatively large, thermal stability may be very poor. In such an instance, six hours of storage at an exaggerated temperature might result in 10%, or more, thermal degradation. With a drug showing such a characteristic, kinetic determinations of the k value at this temperature may result in considerable error if the ETTE concepts are not implemented. The ETTE concepts therefore have their greatest utility in calculations pertaining to shelf life prediction or in selecting suitable storage times when dealing with drugs which have marginal thermal stabilities. Thus, when knowledge of drug thermal sensitivity,

TABLE V.—ETTE VALUES AND PER CENT STORAGE TIME ERRORS FOR A SIX-HOUR OVEN STORAGE TIME AT 75° CALCULATED FOR VARIOUS BOTTLES, CARTONS, AND ACTIVATION ENERGIES

Container	Position	ETTE[a], min. ΔH = 15 Kcal.	ETTE[a], min. 30 Kcal.	ETTE[a], min. 45 Kcal.	% Storage Time Error[a] if ETTE Correction Is Not Made ΔH = 15 Kcal.	% Storage Time Error[a] if ETTE Correction Is Not Made 30 Kcal.	% Storage Time Error[a] if ETTE Correction Is Not Made 45 Kcal.
50 ml.	Central	340	321	312	6	12	15
100 ml.	Central	334	308	296	8	17	22
250 ml.	Central	310	262	240	16	37	50
Carton with 28-ml. bottles	IOC	283	223	193	27	61	87
	SOC[b]	296	245	220	22	47	64
	COC[b]	311	272	254	16	32	42
Carton with 50-ml. bottles	IOC	264	179	140	37	100	157
	SOC[b]	285	217	181	26	66	99
	COC[b]	300	246	221	20	46	63

[a] Calculations based upon room temperature of 25°.
[b] The positional storage error for these positions are:

		15 Kcal.	30 Kcal.	45 Kcal.
28-ml. bottles	SOC	5%	14%	23%
	COC	9%	29%	45%
50-ml. bottles	SOC	11%	34%	56%
	COC	17%	54%	94%

acquired prior to formulation, suggests that 10% or more of the drug may degrade at room temperature in less than two years, the application of ETTE concepts in exaggerated temperature studies assumes increasing importance in prediction of product shelf life. When data are available pertaining to the values of A and ΔH for the drug in question in chemical systems other than the proposed formulation, these may serve as a first approximation of the expected A and ΔH values in the tablets or other new formulations of the drug. Computations may then be made to estimate which combinations of storage temperatures, times, and bottles should be selected to program a thermal drug degradation study which will provide convenient confirmatory data upon which to base a shelf life prediction.

The preceding remarks suggest that the practical approach to the simplification of exaggerated temperature stability studies might be to employ actual oven storage times, (t_2), where time of heating and cooling to equilibrium temperature is short relative to t_2. Equation 19 may be obtained algebraically from Eqs. 6 and 13 to determine the minimum t_2 which will assure that the actual oven storage time will not differ by more than x% from the effective time.

$$\text{Min. } t_2 = [Q/Z_h - t_h - (t_c - t_2)][1 + 100/x\%] \quad \text{(Eq. 19)}$$

Table VI presents figures calculated from Eq. 19 which represent minimum oven storage times at 45, 60, and 75° for commercial bottled tablets where, without ETTE correction, storage time errors would be less than 5%. It should be noted that these figures apply to the experimental conditions of this study, and where ΔH of the Arrhenius equation equals 15 Kcal. Since ΔH will be somewhat variable from one drug to another, it is not possible to establish precise minimum oven storage times, applicable to all tablets, beyond which it is unnecessary to correct for oven storage times. Despite the complications presented by the Arrhenius constants, Table VI is instructive. It suggests that at an oven storage temperature of 75° or lower, actual oven storage times which extend to several days or weeks in bottles of 250-ml. or less capacity would not usually be significantly different from corrected storage times. Also, at the higher storage temperatures, which normally are conducted only for hours or days, one may use the time of Table VI as a standard of reference. Oven storage times at the higher temperatures should be chosen as much in excess of times in Table VI as will permit one to obtain thermal drug degradation data which are amenable to accurate interpretation by chemical kinetic methods. Table VI also suggests that when drug degradation is appreciable after storage for a few hours at elevated oven temperature, advantage in reducing the storage time error is gained by using the smallest bottles and lower storage temperatures. If relatively high storage temperatures seem necessary, an additional step to reduce storage time error might involve the use of Petri dishes, test tubes, or other special containers which facilitate rapid heat transfer.

TABLE VI.—MINIMUM STORAGE TIMES FOR SEVERAL COMMERCIAL BOTTLES OF TABLETS WHICH INSURE STORAGE TIME ERRORS OF LESS THAN 5%[a]

Bottle, ml.	Z_h, (min.$^{-1}$)	Storage Time, hr. 75°C., Q = 4.135	60°C., Q = 3.779	45°C., Q = 3.353
50	0.055	7	3½	Less than
60	0.050	7¾	4	1 hour.
100	0.041	9½	4½	(see text)
120	0.033	11½	5½	
200	0.026	15	7½	
250	0.022	18	9	

[a] Calculated using the expression min. $t_2 = 1/60\{Q/Z_h - t_h - (t_c - t_2)\}[(1 + 100/5)]$. All calculations were done using the hypothetical reaction discussed in Table V, where ΔH = 15 Kcal.

It may be noted in Table VI that numerical values of minimum t_2 at 45° are not given. This is because they are very small values which would never be employed in a practical oven storage stability study at 45°; however, it is of further interest that the ETTE values for samples stored at this temperature, when calculated according to Eqs. 7, 8, and 9, are larger than the actual oven time. This apparently anomalous result occurs because at ΔH =

15 Kcal., the specific velocity constant, k, for thermal degradation at 25° is one-fifth that at 45°. This results in a situation where at any time during cooling, the k of the sample is still an appreciable fraction of the k at 45°, so that the longer one waits for room temperature equilibration, the more significant becomes the contribution of room temperature breakdown to the ETTE. For the degradation presented, where the k at T_r is one-fifth that at T_{ov}, each five minutes spent reaching room temperature adds one more minute to the ETTE. When oven temperature increases, this effect diminishes so that it is negligible at 60 and 75°, but is appreciable near 45° and lower temperatures.

Effect of Product Analysis Time on Shelf Life Prediction.—The realization that the room temperature degradation rate may not be a negligible factor in the calculation of the ETTE points to another possible source of error in estimating effective storage time in stability studies.

In an industrial pharmaceutical laboratory, analyses are normally performed on a priority basis by analytically trained personnel. Scheduling of analytical work and reporting of analytic data may result in delays between an original product analysis and the time when the product is exposed to exaggerated temperatures for stability study. Likewise there may be a delay between the time the product is removed from a stability study oven and the time of assay. Sometimes these combined time lapses may represent several weeks. That is, a sample stored for a specific time in an exaggerated temprature storage oven may have been stored, in fact, an additional several weeks at room temperature. In those cases where the drugs in question have marginal stabilities, this additional time of room temperature storage may have to be included in the calculations for prediction of shelf life. When, for example, the effective oven storage time at 45° is sixteen days, but the product is maintained an additional fifteen days at room temperature prior to analysis because of programming delays, a better estimate of the effective storage time at 45° might be nineteen days. This estimated time would result if the drug degradation rate at room temperature was, for example, one-fifth of that at 45°.

Though the work reported in this paper has been concerned primarily with tablet heating and cooling characteristics under exaggerated temperature stability study conditions and with the way in which these may affect the accuracy of shelf life predictions based on chemical kinetics, the concepts are also applicable to other dosage forms. Short term exaggerated temperature stability studies are assuming increasing importance in pharmaceutical product development. These studies make it possible to select the most stable product formulas early in the course of a product's development. They also give an indication of the shelf life of a product, and thus furnish an intelligent basis upon which a decision for marketing may be based, or upon which reasonable expiration dates may be selected for products of marginal stability.

Selection of stable formulas early in the course of product development permits the use of the correct product formula in the early stages of clinical tests. This is distinctly advantageous since it gives greater assurance that the final marketed product will perform therapeutically in a manner analogous to the results of early clinical evaluation.

The preparation and selection of stable product formulas is normally a development pharmacist's responsibility. The selection of suitably stable formulas and prediction of the product's shelf life based on thermal degradation entails choosing proper exaggerated temperatures, methods, and facilities which assure effective time-temperature storage conditions, packages or containers for the dosage forms, program for withdrawing representative samples for assay, preparation of samples for assay, and finally assay. Despite the frequently high precision of the assay method, the experienced formulator is often faced with assay results of tablets and other dosage forms, which apparently defy the rules of chemical kinetics. Such results may arise, for example, if the original drug isolation and assay method fails to isolate all of the drug from an aged dosage form. Normally such a failure can be easily corrected; however, more subtle reasons for failures that may go undetected result from errors in storage oven heating due to such interrelated factors as, for example, overloading storage ovens, nonuniform temperature within the ovens, improper correlations of stability data derived from products in different sized bottles or cartons, inadequate sampling procedures, or inaccurate estimates of effective storage times and temperatures. Since these factors normally are subject to control by the development pharmacist, it is important that he understands and controls them to the extent that valid thermal degradation data may be obtained when accurate analyses are performed on stability study samples.

More experimental studies which evaluate the importance of each of the numerous factors influencing the collection of thermal drug degradation data would be helpful for improving the procedures employed in drug shelf life prediction.

SUMMARY

1. The experimental work of this study was concerned with the heating and cooling characteristics of tablets in cylindrical bottles and cartons, when these are placed in hot air ovens. Individual tablet temperatures were measured with a thermocouple and a recorder.

2. The heating and cooling rate coefficients for different tablets in a bottle are relatively independent of their positions in the bottle; however, the apparent times for exponential heating to begin are dependent upon tablet position within a given bottle and the oven storage temperature.

3. Apparent exponential heating or cooling in a given bottle occurs sooner in tablets located at the side of a bottle than for those located centrally.

4. For a given bottle and tablets, the heating and cooling rate coefficients were independent of the oven storage temperatures, i. e., the same values were obtained irrespective of whether oven temperatures were 45, 60, or 75°.

5. Bottles used in these studies ranged in volume from 20 to 250 ml. Standard 12-bottle pasteboard cartons were also used. Times required for bottles and cartons of tablets to attain equilibrium temperatures increased with container size. These times thus increased with increased container heat capacity.

6. For given type tablets, heating and cooling rate coefficients decreased with increasing container size.

7. White uncoated tablets showed heating and cooling rate coefficients about 15 per cent higher than gray-coated tablets of similar size, when studies were done in identical bottles. Large pink sugar-coated tablets showed heating and cooling rate coefficients very similar to smaller gray sugar-coated tablets, when studies were done in identical bottles.

8. Under a given set of experimental conditions, where tablet heating was done in a convected hot air oven and cooling was performed on a laboratory bench, heating rate coefficients were always 15–20 per cent greater than the cooling rate coefficients.

9. Heating and cooling rate coefficients are appreciably smaller for bottled tablets in cartons than for similar bottles outside of cartons.

10. Thermal diffusivity interpretations applied to the data suggest that heat transfer through bottled tablets is mediated largely through a radiation process.

11. Using experimentally determined conductivity values in conjunction with bottle dimensional constants, heating and cooling rate coefficients for coated and uncoated tablets are definable with equations. The equations are presented.

12. The ETTE concept was employed to compute the effective storage time of tablets stored under different experimental conditions and these values were compared with actual oven storage times. For example, a 250-ml. bottle filled with large coated tablets and stored for two hundred and forty minutes at 75° has an effective storage time of one hundred and ninety minutes when $\Delta H = 15$ Kcal., and a first-order rate of degradation is assumed.

13. The effects that variables A (probability factor), ΔH, oven temperature, and bottle size have on the computed value of ETTE for a drug in tablets are indicated. Also indicated is how these variables relate to the programming of exaggerated temperature stability studies.

14. A table has been presented giving minimal storage times of different sized bottled tablets at several oven temperatures which can serve as a guide for programming exaggerated temperature stability studies. Use of the table can serve as a guide to the selection of oven temperatures, bottle sizes, and storage times which circumvent the need of ETTE calculations of oven storage time, thereby simplifying calculation of reasonable estimates of product shelf life.

15. It has been noted that the validity of exaggerated temperature stability data may be affected adversely by failure to correct for finite heating and cooling times of samples; also by other interrelated factors such as overloading storage ovens, nonuniform temperature within the ovens, improper correlations of stability data derived from products in different sized containers, and inadequate sampling procedures. Since such factors normally can be controlled by the development pharmacist, it is desirable that he understands the degree of their importance in specific stability studies; also that he exercises the measure of control necessary to give confidence to calculations of product shelf life at room temperature.

GLOSSARY OF TERMS

All temperatures in degrees Kelvin,[4] times in minutes, and dimensions in centimeters.

T_{ov} = oven temperature
T = sample tablet temperature
T_r = room temperature
$T_{90\%}$ = temperature at which the specific breakdown rate equals 0.95 k_{ov}
t = time
t_0 = time when sample is placed in the oven ($t = 0$)
t_1 = time after t_0 for sample to reach oven temperature
t_2 = time after t_0 when sample is removed from oven
t_3 = time after t_0 for sample to reach room temperature again
t_h = time after t_0 for sample at T_{ov} to break down an amount equal to that occurring in a heating sample during its heating cycle (the heating time equivalent)
$(t_c - t_2)$ = time for a sample at T_{ov} to break down an amount equal to that occurring in a cooling sample during its cooling cycle (the cooling time equivalent)
Z_h = specific heating rate (min.$^{-1}$)
Z_c = specific cooling rate (min.$^{-1}$)
f_h = time difference between t_0 and the apparent beginning of logarithmic heating

[4] When temperature differences are measured, i. e., $T_{ov} - T_r$, degrees centigrade may be used, giving values identical to those that result when T values are expressed in degrees Kelvin.

t_c = time difference between t_0 and the apparent beginning of logarithmic cooling
D = diameter of bottle studied
H = height of bottle studied
K = diffusivity constant (cm.²/sec.)
A = kinetic probability constant (10^8 min.$^{-1}$)
ΔH = heat of activation of the breakdown reaction (15, 30, or 45 Kcal./mole)
R = gas constant (1.987 cal. deg^{-1} mole^{-1})
k_{ov} = specific reaction rate at oven temperature (min.$^{-1}$)
ETTE = equilibrium temperature time equivalent (units of time)
Q = (2.303 [log ($T_{ov} - T_r$) − log ($T_{ov} - T_{95\%}$)] (dimensionless)
P_h = numerical solution of the integral of Eq. 8 (dimensionless)
P_c = numerical solution of the integral of Eq. 9 (dimensionless)

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Some Effects of Ionized Air on *Penicillium notatum**

By ROBERTSON PRATT and ROBERT W. BARNARD

Exposure of *Penicillium notatum* to ionized air reduces the production of penicillin, negative ions exerting a greater effect than positive ions. Growth of the mycelium and germination of spores also are depressed, but positive ions exert a greater effect than negative ions on spore germination. Production of carbon dioxide is depressed to about the same extent as mycelial growth.

ABOUT two hundred years ago, the dependence of various biologic functions on atmospheric electricity, both natural and artificially produced, was investigated, and many experiments purporting to show effects upon secretions, blood, pulse, growth, mental activity, and even morality, were described. Disruptive discharge was used therapeutically in Halle, Germany, in 1744 to influence the course of disease, and following description of a method in 1754 to produce positive and negative air ions, aniontherapy was introduced. Sporadic accounts of biologic and therapeutic effects of ionized air have continued to appear through the intervening years, including several reports that an excess of negative ions existing in the atmosphere at some of the most famous spas of Europe is partly responsible for the supposed health-restoring qualities of those resorts. The earlier literature, much of which is in the category of testimonials and speculation, has been reviewed (1). In our own time, negative air ions have been reliably reported to relieve and positive ions to aggravate sinusitis, rhinitis, asthma, and pollenosis in humans (2, 3), and positive air ionization has been reputed to induce headache, dizziness, fatigue, and malaise, while negative air ions have been said to ease the breathing and induce a sense of well being (4). These reports deal with subjective clinical responses.

Objective laboratory research has revealed significant quantitatively measurable effects of ionized air on various biologic systems. For example, exposure to positively ionized air causes a reduction in the succinoxidase content of the adrenal gland of the intact rat (5), both positive and negative air ions are lethal to staphylococci (6), and negative air ions increase and positive air ions decrease or abolish ciliary movement in mammalian trachea, both *in vivo* and *in vitro* (7–9).

This report is concerned with effects of air ionization on certain activities of the fungus, *Penicillium notatum*. Antibiotic production by the mold was chosen as the major activity for study because it is an easily and reproducibly measurable objective index of biochemical activity. The effects of air ionization on spore germination and mycelial growth of the mold and on its carbon dioxide production also were observed.

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AIR IONIZATION

Ionization differs in gaseous and liquid media. Ionization in a liquid results from separation of molecules into anions and cations, whereas in a gaseous medium it results from removal of an electron or substitution with a particle of different electrical charge (10). The ions may be small, consisting of about a dozen gaseous molecules associated with a single electric charge; intermediate, consisting of about 100 submicroscopic molecules bearing a charge; or large, i. e., about 1,000 times the size of small ions (11). The normal atmosphere contains from 500 to 2,000 small and intermediate positive air ions per cm.^{3} and a slightly smaller number of negative air ions (12). The ions range from 0.001 to 0.1 μ in diameter. The chief natural sources of air ionization are cosmic rays and radioactive particles in the air and earth. Such particles are derived mainly from the disintegration of thorium and radium which emit alpha, beta, and gamma radiation.

Air ions may be generated artificially by thermionic methods, or by the use of radioactive materials, X-rays, photoionization, high voltage brush discharges, high frequency currents, and atomization of water, the latter accounting for the high density of negative air ionization in the immediate vicinity of water falls and some spas. These methods have been reviewed (13–15). Accurate measurement of atmospheric electrostatic potential can be made with an ion current probe (16), and effective climate control through regulation of air ionization has been reported (14, 17).

Ion Generator.—The ion generators employed in the present work have a radioactive tritium-titanium foil as a source of beta radiation.[1] The beta rays collide with air molecules and particles and create both positive and negative ions. By means of a rectifier, a unipolar charge is established on a metal plate near the radioactive foil. The plate attracts and traps ions with an opposite charge and repels into the atmosphere ions with a like charge. The velocity of the air ions depends on their size and on the voltage on the rectifier plate. Velocities of the biologically important "small"- and "intermediate"-sized ions vary from about 0.01 to about 2.2 cm./sec./volt/cm. (18). To generate an atmosphere rich in negative ions, the plate is charged negatively. Reversing the polarity will produce a high density of positive ions. Many of the details and much of the theory have been reviewed (15, 19–21).

The foils in the ion generators have an area of about 0.4 cm.^{2}, and each one contains about 50 mc. of tritium. Tritium is a beta ray emitter with an energy of irradiation of 0.015 Mev. The approximate range of radiation at this intensity is 0.002 Gm./cm.^{2} (19). It has been calculated that in air (density 1.2 mg./ml.) the maximum range is 1.7 cm. (6). Since the units were so placed that the target surfaces (*Penicillium* cultures) were 5 cm. distant, the possibility of direct radiation effects on the organism was minimized. Nevertheless, special radiation controls were run in each experiment. At the rectifier voltage employed (860 v.), each unit delivered 10^6 ions per mm.^{2} of culture surface per second. Cultures were exposed continuously throughout each experiment.

[1] The ion generators were kindly furnished by Dr. J. C. Beckett of the Wesix Electric Heater Co., San Francisco, Calif.

EXPERIMENTS AND RESULTS

Antibiotic Biosynthesis.—*Penicillium notatum* ATCC 9178 (NRRL 1249.B21), a surface culture strain of the mold, was used throughout. Technical details of the design of the ionizer equipment made it impractical to study the effect of ionized air on submerged cultures; therefore, surface culture methods were employed. This was considered justifiable, despite the fact that industrial production is by means of submerged culture techniques, since the experiments were aimed at determining whether or not air ions influence biochemical reactions of the mold and not at industrial application. Moreover, because of the low penetrability and the rapid neutralization and dissipation of air ions in aqueous media, it is doubtful that any effect could be observed in submerged cultures even if the available equipment had been adaptable to such an experimental set-up.

The culture medium had the following composition: KH_2PO_4, 0.057 *M*; $MgSO_4 \cdot 7H_2O$, 0.006 *M*; $NaNO_3$, 0.057 *M*; phenylacetic acid, 0.00183 *M*; $ZnSO_4 \cdot 7H_2O$, 0.00015 *M*; lactose, 0.111 *M*; and corn steep liquor solids 2%.

The geometry of the ionizing units limited the choice of glassware for culture vessels. Berzelius beakers (100-ml. size) containing 50 ml. of the above medium plus 2 ml. of inoculum finally were selected. The area of the air-surface interface was 18.1 cm.^{2} and, consequently, the ratio of surface to volume approached 1:3. The yields of penicillin recorded below for control cultures are considerably less than those the organism is capable of producing, but they are not excessively low in view of the relatively unfavorable surface/volume ratio and in view of the light inoculum that was used in order to avoid excessive vegetative growth which would have shielded underlying mycelia from the air ions.

Antibiotic activity was determined by the standard cylinder plate technique, using *Micrococcus pyogenes* var. *aureus* ATCC 6538 P (*Staphylococcus aureus* FDA 209 P), as the test organism.

Data from preliminary experiments indicated that air ionization exerts an effect on the yield of penicillin and that the magnitude of the effect depends on the density of the seeding inoculum and on the duration of the experiment. For example, after seven days of incubation, the yield of penicillin in cultures exposed to negative air ions was reduced about 80% in comparison with nonionized controls when a very sparse inoculum was used, and about 22% when a very dense inoculum was used. The procedure adopted was standardized by using an inoculum of intermediate density which resulted in approximately 50% reduction in yield after seven days. The inoculum consisted of 2 ml. of a spore suspension per 50 ml. of culture medium. The suspension was prepared from three-day-old cultures and was adjusted to 10% transmission as determined by a Lumetron, model 402 EF equipped with a neutral filter.

As a precaution, two controls were run for each experiment, one with no ionizing unit and one a

radiation control to check the effect, if any, of secondary beta radiation. This control consisted of a culture exposed to an ionizing unit which was not connected to the power line. Thus, this culture was exposed to the same radiation as the experimental cultures, but was not exposed to a differential air ionic atmosphere. The ambient atmosphere in this culture vessel was richer in air ions of both polarities, due to the ionizing action of the beta rays. However, in the small confined volume over the cultures, the negative ions could be expected to combine with positive ones, thus tending to restore polar neutrality. That this occurred, and that the results observed in the experimental cultures were due to the air ions and not to a radiation effect *per se*, was indicated by the fact that in all experiments the radiation controls and the standard controls gave identical values within the experimental limits stated in Table I. All experiments were performed in a constant temperature room maintained between 24 and 26°.

Data from five replicated experiments summarized in Table I show that both negative and positive ionization of the air reduced the yield of penicillin, and that negative ions had a more depressing effect than positive ions.

TABLE I.—EFFECT OF NEGATIVE AND POSITIVE AIR IONS ON YIELD OF PENICILLIN AND ON DRY WEIGHT OF MYCELIUM IN CULTURES OF *Penicillium notatum*

Ionization	Penicillin, units/ml.[a] 7 Days	Penicillin, units/ml.[a] 10 Days	Dry Weight of Mycelium, mg.[b] 10 Days
None (control)	73	86	771.1
Negative	38.6	53.9	616.9
Positive	55.8	78	674.7

[a] Maximum deviation among quadruplicate assays in any one experiment ±3%; and among the several experiments ±5%.

[b] Maximum deviation from the average among the several experiments ±7.2%.

It seemed possible, although only remotely so, that the effect on yield of penicillin was not primarily a biological one exerted through the mold, but instead was primarily a physical or chemical one involving destruction of the antibiotic after it was biosynthesized, or perhaps was a combination of the two effects. For example, the reduction in yield of antibiotic activity in cultures exposed to negative ions was considerably greater than might have been expected from the decrease in growth, although it is well known that there is not necessarily a concordance between vegetative growth and antibiotic production in cultures of *Penicillium*. The possibility seemed to exist that equal or nearly equal amounts of antibiotic were produced in all cultures but that the rate of destruction was greater in an atmosphere with a high density of negative ions. Accordingly, sterile filtrate from a seven-day *Penicillium* culture was aseptically apportioned into four sterile culture vessels, one of which was supplied with positively and one with negatively ionized air while the other two served as the usual controls. All were placed in the incubator room. Aliquots were removed aseptically from each vessel daily for seven days and were assayed. The loss in potency was the same in all vessels. Therefore, it seems safe to assume that the ionized air did not have any direct effect on the antibiotic liberated into the culture medium but that the effect was exerted on the fungus.

Comparison of the data for seven days and ten days in Table I suggests that the effect of ionization on yield of antibiotic decreased with time. The penetrating ability of air ions is low. Consequently, only the cells in the mycelium comprising the uppermost surface of the mold mat were exposed, and, therefore, as the thickness of the mold mat increased, an increasing proportion of the mycelial cells was protected from the ionized atmosphere and its detrimental effect on antibiotic synthesis. By the tenth day there was no significant difference in yield from control cultures and those exposed to positive air ions, although the negative ion cultures still showed appreciable depression.

Spore Germination and Mycelial Growth.—Microcultures, consisting of *Penicillium* spores from three-day-old sporulation cultures seeded onto a thin layer of sporulation agar on cover glasses maintained in a humid atmosphere, were exposed to the ionizing units. Five to ten microscope fields, containing 250 to 300 spores, on each slide were examined periodically. There was considerable delay in germination of spores exposed to ionized air of either polarity. For example, at the time (seven hours) when 4% of control spores had germinated, less than 1% germination had occurred in cultures exposed to either negative or positive ionization. After nine hours, 63% of the control spores, 40.7% of the "negative" spores, and 30% of the "positive" spores had germinated. At that time, average germ tube length was 5.2 μ for the control and 4.4 μ for each of the experimental cultures. Observations were not continued beyond nine hours because after that time the growth pattern of the germ tubes made accurate observation and measurement impossible.

The observations on spore germination are consistent with similar studies made on *Neurospora crassa*, in which ionization of either polarity markedly reduced spore germination and positive ionization was found to be more detrimental than negative ionization (22).

The data in Table I show that growth of the mold (as indicated by dry weight of mycelium formed) was reduced approximately 12 to 20% when cultures were exposed to an atmosphere artificially enriched in air ions for ten days.

Carbon Dioxide Production.—Pyrex tubes were annealed to opposite sides of the culture vessels and the vessels were made part of a gas train, being both preceded and followed by potassium hydroxide traps. By applying gentle suction at one end of the train, carbon dioxide-free air was drawn into the culture chamber at a rate of 50 ml. per minute and the carbon dioxide evolved by the cultures was trapped in the succeeding hydroxide tubes. At the end of the experiment, the base in these tubes was titrated against standard HCl to the phenolphthalein end point. Barium chloride was added to remove the soluble carbonate that could act as a buffer. At the end of the run, the mold mats were carefully removed from the culture vessels and dried to constant weight. The titration data were converted to carbon dioxide output per 100 mg. of mycelium.

Values for carbon dioxide produced by control, by "positive," and by "negative" cultures were 159.5 ml., 147.2 ml., and 134.3 ml., respectively, per 100 mg. dry weight of mold over a five-day period. Thus, taking carbon dioxide production of the control cultures as 100%, the corresponding figures for "positive" and "negative" cultures were 92.3% and 84.2%, respectively.

DISCUSSION

It has been shown that spore germination and growth of *Penicillium notatum* and biosynthesis of penicillin are reduced when the organism is exposed to an atmosphere artificially enriched in either negative or positive air ions, but that the effects of the two polarities are not identical and the functions measured are not affected to the same degree.

Whereas negative ionization reduced growth about 20% over a ten-day period, it reduced antibiotic production almost 40% in the same period of time. Such a large difference cannot be ignored. It suggests involvement of at least two different enzyme systems, one of which is more subject than the other to the influence of the ions. It has been established that enzymes can be influenced by air ions. For example, Nielson and Harper (5) showed that when intact rats were maintained in an atmosphere of positively charged air ions for only four hours, the succinoxidase content of the adrenal gland was diminished 13% as compared with control animals. In the present experiments, the mold was exposed continuously for periods up to ten days. Recent work of other investigators suggests that an important target enzyme of air ions is the cytochrome oxidase system (23).

The action of air ionization may affect enzyme systems to produce a molecule with qualitatively (or quantitatively) different antibiotic activity than is normally formed. If this were happening it would not have been detected in the present experiments because assays were performed with only one test organism. This problem should be pursued by the use of chromatographic or other suitable techniques and by bioassays employing multiple test organisms. This is of some interest, since the data on carbon dioxide production, corrected for weight of the mold mat in the different cultures, indicate that respiration proceeds at a lower rate in cultures exposed to ionized air than in the controls. The possibility that air ions alter the permeability of the mycelial cells should not be ignored. Conceivably, nutrients might not be so readily absorbed, thereby, in effect, giving the organism less raw material with which to work or, alternatively, intermediates in the biosynthetic reactions may leach out of the cells before the final antibiotic molecule is formed. In either case lower yields of penicillin might be expected. Further experimentation is needed to evaluate these and other possibilities.

SUMMARY

The literature on air ionization has been reviewed briefly, with particular reference to its biological effects. Although such effects have been reported sporadically for more than two hundred years, many of the reports must be classified as subjective. In recent years the number of reports of objectively demonstrable effects of air ionization on living cells, tissues, and organs has been increasing.

The experiments reported in the present paper showed that air ions of either polarity have a pronounced effect on the ability of *Penicillium notatum* to produce penicillin in surface culture, on the ability of the fungus spores to germinate, and on the vegetative growth of the mold. Behavior of the organism in normal air, i. e., air not carrying a heavier load of one ionic form than the other, was used as a reference for comparison. An atmosphere rich in negative air ions had a greater depressing effect than one rich in positive ions on the synthesis of antibiotic, but, with respect to spore germination, the positive ions were more deleterious than the negative ones.

Preliminary experiments have suggested that carbon dioxide production, corrected for differences in mycelial weight, also is suppressed by both positive and negative air ions and that the negative ions produce a greater depressing effect than the positive ions.

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Rat Bioassay of Combinations of Diuretics*

By MARIO D. G. ACETO† and CASIMIR T. ICHNIOWSKI

A bioassay procedure was used to determine whether or not combinations of acetazolamide sodium with selected diuretic agents would yield more effective responses than those induced by the individual components of a given combination. The diuretics chosen for combination with acetazolamide sodium were: caffeine, mercurophylline, potassium acetate, potassium bicarbonate, potassium citrate, potassium nitrate, sodium acetate, theobromine, and theophylline. The best combinations of relatively weak diuretics were obtained with the acetazolamide sodium + potassium bicarbonate and acetazolamide sodium + potassium acetate series. Both the acetazolamide sodium + caffeine and acetazolamide sodium + potassium nitrate combinations warrant more critical evaluation.

THIS STUDY was undertaken to determine whether or not combinations of acetazolamide sodium[1] with selected diuretics would yield responses better than those induced by the individual components of a given combination. It was also desired to determine if weak doses of two diuretic compounds could be combined and provide a diuresis equal to or greater than that elicited by a larger dose of each component of a combination. If effective responses could be obtained with these combinations such mixtures might offer the advantage of decreased incidence of side effects without loss of desired pharmacologic response.

EXPERIMENTAL

Materials.—The acetazolamide sodium used in these experiments was furnished by Lederle Laboratories.[2] Other diuretics employed were the official products: caffeine, mercurophylline, potassium acetate, potassium bicarbonate, potassium citrate, potassium nitrate, sodium acetate, theobromine, and theophylline. Acacia U. S. P. was used as the suspending agent for the poorly water-soluble caffeine, theobromine, and theophylline.

Procedure.—The general procedure for rat diuretic tests described by Lipschitz, *et al.* (1), was followed. In this study, Wistar strain rats of both sexes in the weight range 110 to 400 Gm. were used. Urine samples were collected in graduated glass cylinders over which were superimposed metal metabolism cages housing the medicated animals. Combinations of two components only were studied. The constant member of each binary mixture was acetazolamide sodium. A 4% acacia mucilage in normal saline was used to prepare stock suspensions of the xanthines. These stock preparations, diluted with an equal volume of saline, were thoroughly shaken just prior to administration. All other solutions were prepared with normal saline as solvent.

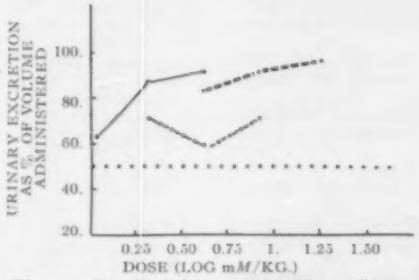

Fig. 1.—Results of acetazolamide sodium + potassium acetate assay A: . . . . ., control; - - - - -, acetazolamide sodium (dose × 10^3); ·-·-·-, potassium acetate; ———, acetazolamide sodium + potassium acetate [doses in the combined curve expressed as mM/Kg. are: (*a*) acetazolamide sodium 0.002 + potassium acetate 1, (*b*) acetazolamide sodium 0.002 + potassium acetate 2, (*c*) acetazolamide sodium 0.002 + potassium acetate 4]. All doses in figure are expressed as log mM/Kg.

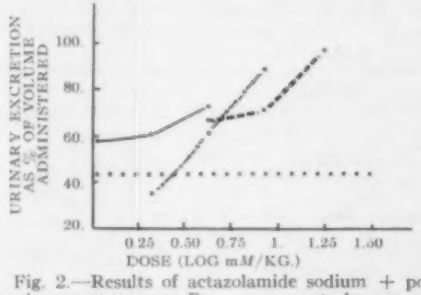

Fig. 2.—Results of actazolamide sodium + potassium acetate assay B: . . . . ., control; - - - - -, acetazolamide sodium (dose × 10^3); ·-·-·-, potassium acetate; ———, acetazolamide sodium + potassium acetate [doses in the combined curve expressed as mM/Kg. are: (*a*) acetazolamide sodium 0.002 + potassium acetate 1, (*b*) acetazolamide sodium 0.002 + potassium acetate 2, (*c*) acetazolamide sodium 0.002 + potassium acetate 4]. All doses in figure are expressed as log mM/Kg.

Eight rats were used for each dose level tested. In any given series of tests, the experimental groups were of approximately equal weight. All administrations were made intragastrically. The

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Abstracted in part from a thesis submitted by Mario D. G. Aceto to the Graduate School of the University of Maryland in partial fulfillment for the degree of Master of Science.
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[1] Diamox Sodium is the registered trade name of Lederle Laboratories for acetazolamide sodium.
[2] The authors express their gratitude to Dr. Christopher H. Demos of the Lederle Laboratories for the generous supply of this material.

TABLE I.—RESULTS OF TWO ASSAYS: ACETAZOLAMIDE SODIUM-POTASSIUM BICARBONATE SERIES

Materials Administered	Dose, mM/Kg.	Urinary Excretion as Per Cent of Volume Administered — Assay A	Assay B
Saline	...	69.2	39.5
Acetazolamide sodium	0.002	62.4	...
Acetazolamide sodium	0.004	59.5	60.5
Acetazolamide sodium	0.008	87.7	67
Acetazolamide sodium	0.017	...	66.4
Potassium bicarbonate	1.1	44.4	...
Potassium bicarbonate	2.2	51.8	...
Potassium bicarbonate	4.4	61.9	...
Potassium bicarbonate	3	...	48.4
Potassium bicarbonate	6	...	62.5
Potassium bicarbonate	12	...	96.8
Acetazolamide sodium	0.002		
+	+	65	...
Potassium bicarbonate	1.1		
Acetazolamide sodium	0.004		
+	+	72.4	...
Potassium bicarbonate	2.2		
Acetazolamide sodium	0.008		
+	+	103.1	87.1
Potassium bicarbonate	4.4		
Acetazolamide sodium	0.004		
+	+	...	96.1
Potassium bicarbonate	4.4		
Acetazolamide sodium	0.017		
+	+	...	100.8
Potassium bicarbonate	4.4		

TABLE II.—RESULTS OF THREE ASSAYS: ACETAZOLAMIDE SODIUM-POTASSIUM NITRATE SERIES

Materials Administered	Dose, mM/Kg.	Urinary Excretion as Per Cent of Volume Administered — Assay A	Assay B	Assay C
Saline	...	38.8	44.8	46.1
Acetazolamide sodium	0.004	46.5	43.2	64.8
Acetazolamide sodium	0.008	66	59.2	74
Acetazolamide sodium	0.017	86.3	68.6	91.1
Potassium nitrate	1.25	45.4	25.4	38.3
Potassium nitrate	2.5	58.9	42.9	59.6
Potassium nitrate	5	93.1	83.1	81.2
Acetazolamide sodium	0.002			
+	+	68.1	58.6	57.7
Potassium nitrate	0.62			
Acetazolamide sodium	0.004			
+	+	94.5	...	...
Potassium nitrate	1.25			
Acetazolamide sodium	0.008			
+	+	109.7	...	...
Potassium nitrate	2.5			
Acetazolamide sodium	0.002			
+	+	...	42.1	52.3
Potassium nitrate	1.25			
Acetazolamide sodium	0.002			
+	+	...	65.3	63.2
Potassium nitrate	2.5			

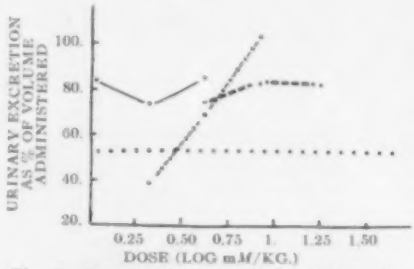

Fig. 3.—Results of acetazolamide sodium + potassium acetate assay C: ....., control; - - - - - -, acetazolamide sodium (dose × 10^3); ·····, potassium acetate; ———, acetazolamide sodium + potassium acetate [doses in the combined curve expressed as mM/Kg. are: (*a*) acetazolamide sodium 0.002 + potassium acetate 1, (*b*) acetazolamide sodium 0.002 + potassium acetate 2, (*c*) acetazolamide sodium 0.002 + potassium acetate 4]. All doses in figure are expressed as log mM/Kg.

TABLE III.—RESULTS OF THREE ASSAYS: ACETAZOLAMIDE SODIUM-CAFFEINE SERIES

Materials Administered	Dose, mM/Kg.	Urinary Excretion as Per Cent of Volume Administered — Assay A	Assay B	Assay C
Saline	...	40.8	43.5	55.2
Acacia in saline	...	...	...	56.9
Acetazolamide sodium	0.004	43.5	80.8	80.2
Acetazolamide sodium	0.008	56.5	67.6	66.3
Acetazolamide sodium	0.017	61.2	85.6	100
Acetazolamide sodium in acacia-saline	0.004	...	...	62
Acetazolamide sodium in acacia-saline	0.008	...	...	82.8
Acetazolamide sodium in acacia-saline	0.017	...	...	87.6
Caffeine	0.1	55.9	70.5	74.7
Caffeine	0.2	44.1	52.5	79.9
Caffeine	0.4	61.2	76.9	73.9
Acetazolamide sodium	0.008			
+	+	91.1	...	...
Caffeine	0.1			
Acetazolamide sodium	0.008			
+	+	87.6	...	...
Caffeine	0.2			
Acetazolamide sodium	0.008			
+	+	107.7	...	...
Caffeine	0.4			
Acetazolamide sodium	0.002			
+	+	...	48	...
Caffeine	0.1			
Acetazolamide sodium	0.002			
+	+	...	77.1	...
Caffeine	0.2			
Acetazolamide sodium	0.002			
+	+	...	104.9	117.7
Caffeine	0.4			

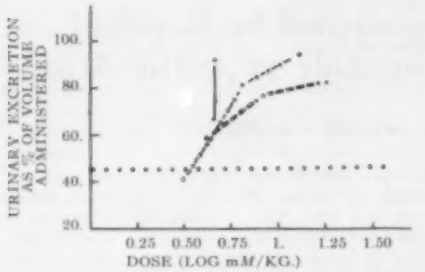

Fig. 4.—Results of acetazolamide sodium + potassium bicarbonate assay C: ....., control; - - - - - -, acetazolamide sodium (dose × 10^3); ·····, potassium bicarbonate; ———, acetazolamide sodium + potassium bicarbonate [doses in the combined curve expressed as mM/Kg. are: (*a*) acetazolamide sodium 0.002 + potassium bicarbonate 4.4, (*b*) acetazolamide sodium 0.004 + potassium bicarbonate 4.4, (*c*) acetazolamide sodium 0.008 + potassium bicarbonate 4.4]. All doses in figure are expressed as log mM/Kg.

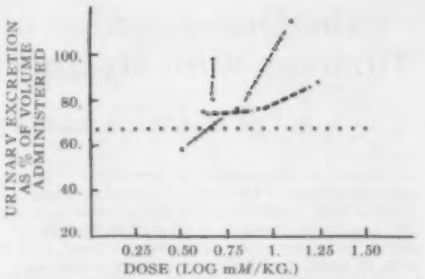

Fig. 5.—Results of acetazolamide sodium + potassium bicarbonate assay D: ....., control; - - - - - -, acetazolamide sodium (dose × 10^3); ·····, potassium bicarbonate; ———, acetazolamide sodium + potassium bicarbonate [doses in the combined curve expressed as mM/Kg. are: (*a*) acetazolamide sodium 0.002 + potassium bicarbonate 4.4, (*b*) acetazolamide sodium 0.004 + potassium bicarbonate 4.4, (*c*) acetazolamide sodium 0.008 + potassium bicarbonate 4.4]. All doses in figure are expressed as log mM/Kg.

volume administered to each animal was 25 cc./Kg. Dose-effect relationships were established for each diuretic material. Based on these data, combinations of acetazolamide sodium with other individual diuretics were prepared and screened for effect. The promising combinations were then subjected to further study. An estimate of the activity of a diuretic combination included, on any given day of the test, a comparison of the dose-response relationships of the combinations with those of the individual components of the combination.

RESULTS AND DISCUSSION

The two series considered the most effective combinations of weak diuretic doses were acetazolamide sodium with potassium bicarbonate and the carbonic anhydrase inhibitor with potassium acetate. The various interrelationships in these two series are given in Figs. 1 to 5. The slopes representing the acetazolamide sodium + potassium bicarbonate dose-effect relationships in Figs. 4 and 5 appear perpendicular because, for each dose combination, the dose for potassium bicarbonate remained constant at 4.4 mM/Kg. and the dose for acetazolamide sodium varied only from 0.002 to 0.008 mM/Kg. Table I presents additional similar data obtained from other assays in the acetazolamide sodium + potassium bicarbonate series not presented in graph form.

Although certain dose combinations in both the acetazolamide sodium + potassium nitrate and acetazolamide sodium + caffeine series indicated merit in combining weak doses of the components, as shown in Tables II and III, respectively, it is felt that the limited testing precludes a general conclusion.

Tests carried out with potassium citrate, sodium acetate, theophylline, and theobromine were indicative that these diuretics offered little promise when combined with the carbonic anhydrase inhibitor.

Attempts to include mercurophylline in this study were unsuccessful. Inconsistent results were obtained with doses ranging from 0.01 to 81.0 mg./Kg. Even when the assay time was extended to eight hours to determine any delayed action of the mercurial, no increase in diuretic effect occurred.

With regard to the xanthine diuretics, the inconsistencies and difficulties encountered by Lipschitz, *et al.* (1), in the main were confirmed.

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The Determination of Eucalyptol by Residual Titration With Hydrogen Bromide in Acetic Acid*

By MARTIN I. BLAKE† and GILBERT RABJOHN

Eucalyptol is determined by treating samples with an excess of hydrogen bromide reagent. After a forty-eight-hour standing period, excess hydrogen bromide is titrated with sodium acetate in acetic acid. Mixtures of eucalyptol with thymol, menthol, and camphor are analyzed for eucalyptol content. Quantitative recoveries are reported.

THE OFFICIAL compendia have never recognized an assay procedure for eucalyptol as the free compound, or in the form of eucalyptus oil, or when combined with other medicaments. The British Pharmacopoeia (1) adopted a method of analysis based on the melting point of the molecular compound which forms with *o*-cresol. Guenther (2) describes a freezing point method. Martin and Harrisson (3) developed a spectrophotometric procedure based on the color reaction with *p*-dimethylaminobenzaldehyde. Methods for the detection and determination of eucalyptol were reviewed by these authors.

Durbetaki (4) used hydrogen bromide in acetic acid for the direct visual titration of oxirane oxygen in epoxy type compounds. The procedure was shown to be accurate and rapid. This paper describes a modification of this procedure for determining eucalyptol, an inner ether. The sample is treated with an excess of hydrogen bromide reagent and after the reaction is complete, unreacted hydrogen bromide is titrated with sodium acetate in acetic acid using methyl violet as the indicator. The procedure is applied to mixtures of eucalyptol with menthol, camphor, and thymol.

EXPERIMENTAL

Preparation and Standardization of Solutions.—Hydrogen bromide 0.1 *N* in acetic acid was prepared (5) by adding bromine dropwise into tetrahydronaphthalene (Tetralin). The hydrogen bromide gas, which formed as a result of the reaction, was passed into glacial acetic acid. A concentrated solution of hydrogen bromide in acetic acid was prepared in this way and corresponded to approximately a 0.5 *N* solution. Sufficient glacial acetic acid was added to make the solution about 0.1 *N*. The final solution was standardized by titration with 0.1 *N* sodium acetate in acetic acid. This was accomplished by transferring exactly 25 ml. of hydrogen bromide solution by pipet to a 125-ml. Erlenmeyer flask. Twenty milliliters of glacial acetic acid was added, followed by the addition of three drops of methyl violet indicator solution (prepared by dissolving 1.0 Gm. methyl violet crystals in 100 ml. of glacial acetic acid). A stirring bar was added and the solution, magnetically stirred, was titrated to the first permanent blue color with 0.1 *N* sodium acetate in acetic acid.

Sodium acetate 0.1 *N* solution was prepared and standardized as described in an earlier paper (6).

Procedure of the Analysis of Pure Eucalyptol.—A sample of eucalyptol, 100–200 mg., was accurately weighed into an iodine flask. This was best accomplished by placing eucalyptol into a half-ounce dropper bottle and weighing the system. Eucalyptol, 5–8 drops, was added to the flask and the system was again weighed. The sample weight was obtained from the difference in weight. Twenty milliliters of glacial acetic acid was added to the flask, followed by the addition of exactly 25 ml. of 0.1 *N* hydrogen bromide solution. The flask was tightly stoppered and permitted to stand at room temperature for forty-eight hours. Excess hydrogen bromide was determined by rapidly titrating with 0.1 *N* sodium acetate solution to the first permanent blue color using three drops of methyl violet indicator solution for detection of the end point. The solution was magnetically stirred during titration. A blank run was conducted in the same manner except no eucalyptol was added. With each series of 4–6 determinations a blank was run to correct for any changes in normality of the hydrogen bromide solution. The analysis of pure eucalyptol is reported in Table I.

TABLE I.—ANALYSIS OF PURE CONSTITUENTS[a]

Constituent	Recovery, %
Eucalpytol	99.86 ± 0.11
Camphor	00.00
Thymol	00.00
Menthol	00.00

[a] Eight determinations.

Calculation of Per Cent Recovery.—Per cent recovery of eucalyptol was calculated from the expression

$$\frac{\left[\begin{array}{l}\text{Vol. 0.1 } N \text{ acetate} \\ \text{consumed in blank}\end{array} - \begin{array}{l}\text{Vol. 0.1 } N \text{ acetate} \\ \text{consumed in run}\end{array}\right] \times 7.713 \times 100}{\text{Weight of sample in mg.}} = \% \text{ Eucalyptol}$$

Reaction Time.—The course of reaction between eucalyptol and hydrogen bromide was studied by analyzing a series of eucalyptol samples after stand-

* Received September 18, 1959, from the School of Pharmacy, North Dakota Agricultural College, Fargo.

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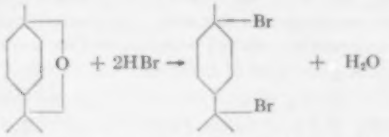
ing for varying time intervals. Reaction time varied from one to one hundred and twenty-eight hours. Reaction was found to be complete after standing at least forty hours. Forty-eight hours was selected as convenient time for this procedure.

Analysis of Eucalpytol Mixtures.—Constituents commonly employed with eucalyptol were analyzed in the manner described for eucalpytol. These included thymol, menthol, and camphor. Results are reported in Table I.

Mixtures containing eucalyptol and one or more other constituents were analyzed. These were prepared by weighing calculated quantities of the components into a 50-ml. volumetric flask and diluting to the mark with anhydrous methanol. Analysis was effected by transferring a 1-ml. aliquot of the solution by pipet to an iodine flask and treating in the same manner as the pure eucalyptol samples. At least four 1-ml. aliquots and a blank were run simultaneously on each mixture. Representative mixtures are indicated in Table II. The components are listed in the first column and their concentrations in mg. per ml. are shown in column 2. Per cent recoveries are indicated in the third column.

DISCUSSION

Eucalyptol has been shown to react quantitatively with hydrogen bromide in acetic acid. The equation for the reaction appears to be



Since the reaction was found to be a slow one, direct titration was not feasible. However, good results were obtained by adding an excess of hydrogen bromide reagent and permitting the reaction to stand for a forty-eight-hour period. The excess hydrogen bromide was readily determined by titration with sodium acetate in acetic acid using methyl violet as the indicator. No difficulty was encountered in observing the first blue color denoting the end point. Since each mole of eucalyptol reacts with two moles of hydrogen bromide, the equivalent weight of the former is one-half the molecular weight or 77.13. Therefore, as shown in the expression for calculating per cent eucalyptol, each ml. of 0.1 *N* sodium acetate is equivalent to 7.713 mg. of eucalyptol. Quantitative recovery is shown in Table I. Since no reaction was observed with thymol, menthol, or camphor, the procedure described in this paper is suitable for determining eucalyptol in the presence of these constituents. Results of the analysis of a variety of such mixtures are shown in Table II.

TABLE II.—ANALYSIS OF MIXTURES FOR EUCALYPTOL CONTENT

Constituents in Mixture	Concentration, mg./ml.	Eucalyptol Recovery, %
Eucalyptol	138.0	99.12 ± 0.22
Camphor	50.4	
Eucalyptol	140.3	101.33 ± 0.31
Thymol	51.5	
Eucalyptol	139.5	99.01 ± 0.16
Menthol	49.0	
Eucalyptol	152.8	98.75 ± 0.25
Menthol	20.0	
Camphor	19.9	
Eucalyptol	151.6	99.45 ± 0.23
Menthol	40.2	
Thymol	39.8	
Eucalyptol	139.3	98.86 ± 0.19
Menthol	20.2	
Camphor	19.8	
Thymol	20.0	
Eucalyptol	143.4	101.85 ± 0.12
Menthol	39.8	
Camphor	40.1	
Thymol	41.2	

The procedure was applied to the analysis of eucalyptus oil for eucalyptol content. A series of ten determinations, based on the procedure described for eucalyptol, yielded an average result of 85.21%. However, by the British Pharmacopoeia method (1) an average result of 71.5% was obtained. The freezing point method, described by Guenther (2), gave an average result of 72.0%. Apparently, other constituents present in the oil, probably unsaturated compounds, reacted with the hydrogen bromide, producing high results. Other work conducted in this laboratory indicated that this reagent reacts quantitatively with certain unsaturated volatile oil constituents. This will be reported in another paper.

The hydrogen bromide titrant must be stored in a tightly stoppered container. It was noted that over a period of several weeks, the normality of the solution decreased slightly. This was taken into account by conducting a blank with each series of determinations.

The procedure described in this paper is a simple and accurate one. The chief disadvantage is the forty-eight-hour standing period required for completion of the reaction. The reaction appears to be very selective, affecting specific types of ethers and certain unsaturated compounds. Basic compounds such as amines also interfere.

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The Toxicity and Safety Testing of Disposable Medical and Pharmaceutical Materials*

By JOHN H. BREWER and HAROLD H. BRYANT

Many disposable surgical and pharmaceutical materials are not adequately tested for their toxicity and compatibility with tissues. Suggested techniques are described for testing these items. By the use of such tests the various items available may be checked and a choice made between those which should not be used and those which have been adequately tested and are found to be satisfactory.

BECAUSE of high labor cost and an inadequate supply of properly trained personnel, the demand for disposable, ready-to-use medical and pharmaceutical materials has resulted in the introduction of many such items in the commercial market. Disposable syringes, needles, vials, catheters, drain tubes, and countless other items are now available. For the most part these are made of plastic or one of the newer, lighter weight alloys. Many of these items offer advantages over the original re-usable item. The throw-away hypodermic needle, for instance, cannot transfer infectious hepatitis from one patient to another, and, since the needle point does not have to withstand repeated injection, it can be made much sharper. More care can be given to its sterilization in autoclaves or gas sterilizers with the proper controls than by the busy physician with an office sterilizer. Also, the individual needle can be sealed in the final package before sterilization, and it maintains sterility until the time of use.

Other disposable items have similar advantages over the original re-usable ones. By changing the plasticizers or other chemicals in the plastic formulations, catheters may be made with any flexibility desired. From a pharmaceutical point of view, bottles and pharmaceutical "glassware" which will not break have many advantages. The pharmacist must take care, however, in the use of these materials, since some of the plasticizers may be leeched out and react with the drugs stored in them. Or they may be permeable to, or soluble in, the solvents which they contain.

The individual plastic or alloy formulations must be carefully tested by chronic and acute testing methods before being employed. Tests which simulate actual conditions of use should be employed, with added tests to give a large degree of safety.

The responsibility of the manufacturer does not end with the careful checking of the original formulation, but tests on each individual lot must be performed, since plasticizers, coloring agents, and plastics may change from lot to lot without the manufacturer's knowledge.

The Food and Drug Administration has been very interested in those plastic formulations used for food containers, and a number of publications have appeared stating which plasticizers and other substances may or may not be used for such items. This has led to some false sense of security on the part of the surgical suppliers; they felt that since their formulations met these requirements that they were entirely satisfactory and met all the requirements for medical use. A great deal of harm may have been done by the labeling of some plastic tubings as medical grade tubing, without adequately testing the material by imbedding samples in animals. Drains and indwelling catheters made from some of this material cause tissue reaction and necrosis which is open to bacterial infection.

Although the U. S. P. has certain sterility and toxicity requirements for plastic tubing assemblies used in blood collection and transfusion, no such requirements or tests are outlined by either the U. S. P. or FDA for tubing to be used for drains or indwelling catheters.

One has only to recall the reports in publications of the AMA on reactions to improperly tested plastic formulations used in watch bands, belts, etc., to understand how much more important is the adequate testing of plastics for surgical and medical use.

Several years ago the authors were asked by a firm, which was planning to manufacture a plastic-hubbed hypodermic needle, to develop toxicity and safety tests for this item. The procedure devised would be used to determine whether the plastic formulations, which met their physical specifications, would also prove nontoxic under all the uses to which needles would be subjected. Several arbitrary tests were developed; some

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This report deals with the development of routine methods which are applied to products of the Becton, Dickinson Co., Rutherford, N. J., and is reported with the permission of that Company.

Presented to the Scientific Section, A. PH. A., Cincinnati meeting, August 1959.

actually simulated conditions of use and others were developed to give a margin of safety. It was found that a few coloring materials could not be used since they would leech out on long contact with saline, which might occur during use. Some formulations were found to be toxic and could not be used. In like manner, some plasticizers were found to be unsatisfactory.

TEST PROCEDURES

The specific tests which are applied to needles are as follows: Each needle hub formulation is checked, using the following solvents: 0.9% saline, 5.0% ethyl alcohol, sesame oil, and polyethylene glycol, since these would represent the most common solvents used in parenteral products. Not less than 20 needles are covered with each of the above menstrua, 1.5 ml. per needle. The overlay preparations, along with control portions of the four solvents, are held at 37° for eighteen to twenty-four hours. The eluates are decanted after vigorous agitation to aid elution of possible surface reaction products. The following tests are then performed:

Acute Toxicity.—Fifteen healthy mice, weighing 18–22 Gm., are injected with each of the menstrua, paralleled by five mice using the control solutions. The saline and ethyl alcohol eluates are injected intravenously, 1.0 ml. per mouse. The sesame oil and polyethylene glycol are injected intraperitoneally, 1.0 ml. per animal of the first, while a dilution of the polyethylene glycol is used which is just below the level causing toxic manifestations in previous control studies. The animals are observed for seventy-two hours.

Bleb Reactivity.—Each of the four elution menstrua is injected intracutaneously, 0.2 ml. into each of ten sites, paralleled by ten 0.2-ml. control blebs in the backs of two rabbits. The first three are injected undiluted, while the polyethylene glycol is diluted to contain 20 mg. in the 0.2-ml. bleb volume. The sites are observed for seventy-two hours for erythema, edema, or necrosis.

Pyrogenicity.—Ten needles are heated in 200 ml. saline to not less than 85° for one hour and the U. S. P. pyrogen test on the decanted eluate performed.

After the original formulation study, production control tests are run, using the saline overlay only.

To simulate actual conditions of use, as in dermatological work, needles are attached to a small syringe and 0.25 ml. saline is drawn into the syringe, leaving the needle cannula filled. These are placed in a tray and held twenty-four hours at 37°, and then blebs raised on a rabbit. Since only a very small area of the inside of the hub is actually in contact with the solution, one might never expect to obtain any toxic reaction. In this manner it has been possible to observe toxicities on some of the formulations discussed above.

OBSERVATIONS

Although these tests were designed for plastic hubs, some interesting findings were noted when experimental lots of aluminum hub needles were submitted to these tests. At first it was not thought necessary to test an all metal needle for toxicity, since no plasticizers or epoxy resins were involved. Since it was known that some of the alloys used contained a small amount of lead to facilitate manufacture, we decided to submit these needles to the same test. A floccular precipitate developed in the saline, and on intravenous injection into the mice killed all of the ten mice within a few minutes. When this floccular material was submitted to the bleb type of test, all the test sites gave positive skin reactions.

These sudden deaths and positive skin tests could not possibly be due to the small amount of lead eluted from these hubs since less than 1 mg. of weight loss occurred during the saline soaking. The deaths must be due to embolism of the floccular material which occurs with aluminum and saline. These tests were repeated with distilled water, and only a slight haze was seen. However, when this material was made isotonic with sodium chloride for intravenous injection, the floccular precipitate developed and death resulted in the mice, although not to the same extent since the amount of precipitate was not as great. No deaths occurred when this material was given intraperitoneally or when the experimenter was very careful not to disturb the precipitate and only injected the supernatant liquid.

This type of safety test does not simulate actual conditions of use since the entire surface area is in contact with the solution. In the actual use type of test in which the needles were placed on the syringe and 0.25 ml. saline drawn into the syringe and allowed to stand, as might occur in a dermatologic clinic where large numbers of allergens are being tested, positive skin tests did occur in the blebs raised on the rabbits, provided the material was mixed by shaking and the precipitate which formed expelled. To determine whether or not this material might also cause false positive reactions in humans, dermatologists repeated these tests, making sure that they mixed the material before injection. About 40% false positive reactions on two series of tests employing this technique were obtained. The number of reactions in the animals and humans is proportional to the amount of precipitate which is injected. If the syringe is handled very carefully so that most of the precipitate remains in the syringe, fewer positive reactions will result.

From the point of view of the hospital pharmacist or laboratory technician, aluminum-hubbed needles should not be used on a pitkin or other type dispensing syringe, which is placed back in the flask of saline each time in order to fill it and then remains in the saline. When used in blood grouping or other tests employing washed red cells, the small amount of aluminum eluted may cause agglutination of the red cells and lead to erroneous results. This finding was noted by one of the blood banks that was using saline from a bottle to which an aluminum needle was attached and which remained in contact with the saline for a long period of time.

For disposable syringes a slightly different technique must be employed in testing. Each syringe formulation is studied, using the solvents discussed under needle testing. With the syringes, the barrels of 20 units are filled with the solvent and allowed to warm to 37° for twenty-four hours. The eluates are then combined and tests for acute toxicity and

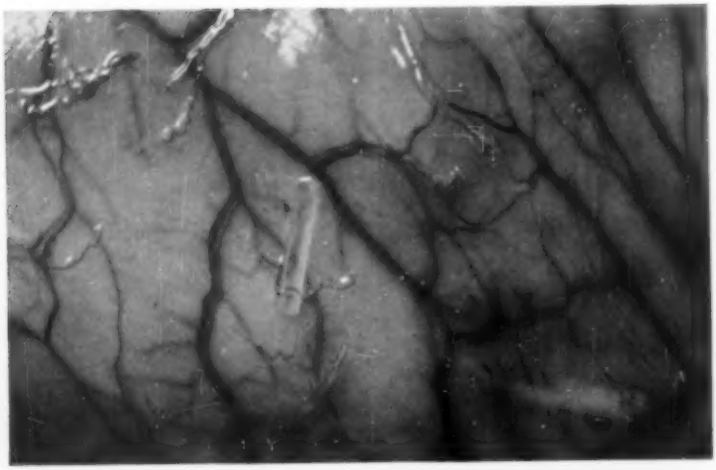

Fig. 1.—Subcutaneous implant; seventy-two hours, no reaction.

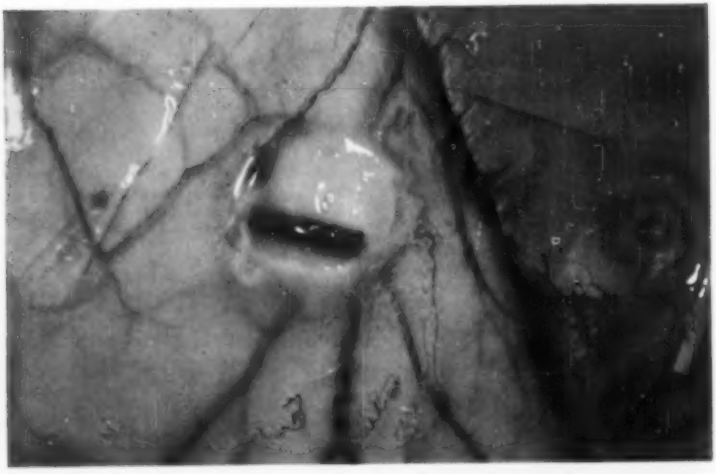

Fig. 2.—Subcutaneous implant; seventy-two hours, showing reaction.

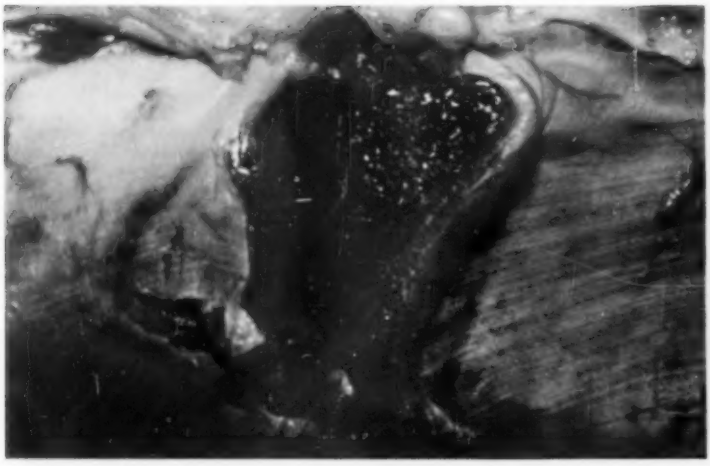

Fig. 3.—Intramuscular implant; seventy-two hours, no reaction.

Fig. 4.—Intramuscular implant; seventy-two hours, showing reaction.

bleb reactivity simulating those for the needle eluates are performed.

Plastic syringe plungers and rubber plunger tips are studied by elution, in much the same manner as described above for needles, except that 10 Gm. of the test material may be covered with 50 ml. of the elution menstruum rather than by using 20 test items.

Control testing of approved formulations in syringe assemblies is performed by drawing 1.0 ml. of 0.9% saline into the syringe plus 1.0 ml. of air, and eluting the assembly for eighteen to twenty-four hours at 37°. Twenty units are so eluted and the eluates combined for acute toxicity and bleb reactivity tests.

Other plastic items: indwelling catheters, drains, stomach tubes, intravenous feeding tubes, and tubing for general medical use are also tested. Plastic formulations developed for use in contact with body tissues, especially where the normal epithelium has been interrupted, are tested for acute toxicity, bleb reactivity, tissue reaction, and pyrogenicity.

Ten grams of the material is cut into relatively small pieces and covered with 50 ml. of sterile 0.9% saline; a like preparation is made using 5.0% ethyl alcohol. These overlays are then warmed to 37° for twenty-four hours, along with control portions of the two menstrua. The overlays are then vigorously shaken and the elution menstruum decanted.

Acute toxicity is determined by injecting the undiluted eluates and control solution in doses up to 1.0 ml., both intravenously and intraperitoneally, in mice weighing 18–22 Gm. This is equivalent to injecting the eluate from 10 Gm. of the plastic per Kg. of body weight.

Bleb reactivity is determined in a manner simulating that described above under needle testing.

Tissue reaction or toxicity exhibited by the tissues in contact with the plastic material is studied by implanting sections roughly 1.5 × 10 mm. in size subcutaneously and intramuscularly in the rabbit. At least four such sections are implanted subcutaneously, paralleled by four known nonreactive controls; a like number are implanted intramuscularly in a second animal. The implants are allowed to remain in place for seventy-two hours, after which the animals are sacrificed and the implant sites examined for surrounding reactive material. The acute, seventy-two-hour tests are evaluated by careful gross examination only. Those formulations which pass the acute implant tests and which may be in contact with the body tissues for a longer period of time are also implanted for sixty-day periods. These implant sites are then evaluated by gross examination and histologically by a competent clinical pathologist. Likewise, those formulations likely to be used in contact with the central nervous system are implanted for seventy-two hours and sixty days in the cerebral cortex of the rabbit for careful evaluation. The implantation procedure has proved to be the most useful of the tests devised to determine tissue compatibility of a plastic formulation.

Pyrogenicity is studied by heating 10 Gm. of the formulation in 200 ml. of saline to not less than 85° for one hour, and subjecting the eluate to the standard U. S. P. pyrogen test procedure.

When a number of the medical grade tubings were tested, some were found to be entirely satisfactory and produced no reaction in contact with the tissues, as shown in Figs. 1 and 3. Other samples of tubing produced a very severe reaction, as shown in Figs. 2 and 4.

SUMMARY AND CONCLUSIONS

It is felt that before use of any material which may produce pathological conditions, that unless there is proof that such testing has been done on each lot produced, similar tests on each lot of material purchased should be run.

As stated above, some of these tests are severe and do not simulate actual conditions of use, but were designed to give a margin of safety. They may also explain some of the reactions which have been encountered in the use of these "throw-away" type products which, until now, could not be accounted for.

It is realized that the tests described are not ideal for all of the different disposable items available. The purpose of this paper is to stimulate the hospital pharmacist or other persons responsible for the purchase of disposable medical supplies to make sure that adequate sterility and toxicity testing has been performed, and that the items are satisfactory for the purpose for which they are to be employed.

Effects of Tranquilizers on Bacterial Toxemias II. Meprobamate*

By LEO GREENBERG and JAMES W. INGALLS

Pretreatment of rats and mice with meprobamate prior to inoculation with lethal doses of bacterial exotoxins and endotoxins resulted in a highly significant prolongation of life. However, the drug showed no effect on survival time in overwhelming infections induced by *Diplococcus pneumoniae* or *Salmonella typhimurium*. In *Salmonella enteritidis* infection, chlorpromazine increased the susceptibility of mice far more markedly than did meprobamate.

MEPROBAMATE is a potent pharmacological agent of wide clinical use, and its role in the alleviation of stress syndromes of various etiologies is well known. Yet, few references to its role in bacterial stress have been reported. It is known that the drug is capable of significantly prolonging the life of guinea pigs in diphtheric toxemia (1), and recent publications have indicated that it significantly affects the immunological response of rabbits to *Salmonella typhimurium* infection (2, 3).

In a previous study (4), it was shown that reserpine is capable of favorably influencing the survival time of experimental animals injected with lethal doses of tetanus toxin, botulinum toxin, or *Diplococcus pneumoniae*. On this basis, our experiments were extended to other psychotropic drugs within the framework of bacterial stress. The present paper summarizes our results with meprobamate.

MATERIALS AND METHODS

The animals used in this study were 200–300 Gm. male and female CFN rats and 20–25 Gm. CFI male mice. All were caged in small groups and kept in the thermostatically controlled animal house for several days prior to use. In toxin experiments, food was withheld after inoculation, but water was given *ad libitum*.

Meprobamate[1] powder was dispersed in 2.5 or 5.0% acacia solution. In all experiments, paired groups of animals were used. The control group was injected with acacia solution in a volume equal to that received by the experimental group. All control and experimental animals in any given series were inoculated within a few minutes of one another.

Tetanus toxin[2] in 0.85% sodium chloride solution with 0.3 *M* glycine, an Lf titer of 870/ml. and a mouse LD_{50} of 112 million was used throughout. Purified *Clostridium botulinum* type A toxin[3] diluted with two parts of glycerol and a mouse LD_{50} of approximately 5×10^8/ml. was used. Toxins were stored in the refrigerator, the botulinus material at −20° to maintain potency. *Escherichia coli* 026:B6 and *Salmonella typhosa* 901 endotoxins were purchased as powdered lipopolysaccharides from Difco Laboratories. All toxins were diluted with sterile isotonic saline.

Diplococcus pneumoniae strain SVI was obtained as a lyophilized culture from the Rockefeller Institute and grown in brain heart infusion broth (Difco). Slants were maintained on brain heart infusion agar with added blood. Subculturing was done weekly and virulence maintained by monthly mouse passage. For inoculations, eighteen-hour broth culture containing approximately 2×10^8 organisms/ml. was used.

Salmonella enteritidis (No. 13076) and *Salmonella typhimurium* (No. 6994) cultures were purchased from the American Type Culture Collection and maintained on Trypticase soy agar (Difco) with added 1% glucose. An eighteen-hour Trypticase soy broth culture containing approximately 4×10^8 organisms/ml. was used for inoculations.

All toxin and bacterial injections were in a volume of 0.1 ml. All inoculations were made intraperitoneally. None of the diluent substances used showed any toxicity to mice or rats, and sterile broth injections were without apparent effect.

RESULTS AND DISCUSSION

Toxin Studies.—Results with meprobamate at various dose levels in both rats and mice subjected to lethal doses of bacterial toxins are summarized in Table I. It is evident that in doses between 200 mg./Kg. and 533 mg./Kg., meprobamate exerted a powerful life-prolonging effect in both species. This effect was demonstrated in both exotoxic and endotoxic states, and in all cases was directly proportional to the dose of meprobamate used.

As in our previous findings with reserpine (4), this response was evoked only under the specific set of conditions which were employed, namely, a large dose of meprobamate injected shortly before the introduction of a highly lethal dose of toxin. The failure of the drug to influence survival when administered one hour post-toxin is apparent. Further, no influence on survival was noted following the use of highly dilute toxin solutions and the use of small divided doses of meprobamate prior to toxin injection.

The studies on *E. coli* and *S. typhosa* endotoxins were carried out several weeks apart. The remarkable similarity of the data achieved apparently sup-

* Received March 14, 1960, from the Research Institute of the Brooklyn College of Pharmacy, Long Island University, Brooklyn 16, N. Y.

[1] Supplied as Miltown through the courtesy of Wallace Laboratories, New Brunswick, N. J.

[2] Supplied through the courtesy of Dr. H. A. Dettwiler, Eli Lilly & Co., Indianapolis, Ind.

[3] Supplied through the courtesy of Matteo Cardella, Immunology Branch, U. S. Army Biological Warfare Laboratories, Fort Detrick, Frederick, Md.

TABLE I.—EFFECT OF MEPROBAMATE ON SURVIVAL TIME FROM BACTERIAL TOXINS IN MALE RATS AND MICE

Animal	No.	Treatment	Dilution	Survival, min. ± S. E.	Significance
		Botulinus Exotoxin			
Mice	10	533 mg./Kg. Meprobamate	1-100	135.0 ± 11.9	$P < 0.05$
Mice	10	Acacia solution	1-100	102.2 ± 8.9	
Mice	25	533 mg./Kg. Meprobamate	1-10	92.0 ± 7.9	$P < 0.01$
Mice	25	Acacia solution	1-10	65.9 ± 2.9	
Rats	7	500 mg./Kg. Meprobamate	1-10	515 ± 50	$P < 0.01$
Rats	7	Acacia solution	1-10	348 ± 15	
Rats	10	400 mg./Kg. Meprobamate	1-10	437 ± 28	$P < 0.01$
Rats	10	Acacia solution	1-10	323 ± 11	
Rats	10	400 mg./Kg. Meprobamate post-toxin	1-10	255	No significance
Rats	10	Acacia solution post-toxin	1-10	258	
		Tetanus Exotoxin			
Mice	10	533 mg./Kg. Meprobamate	1-100	234.5 ± 20.7	$P < 0.05$
Mice	10	Acacia solution	1-100	180.0 ± 12.7	
Rats	10	500 mg./Kg. Meprobamate	1-10	1057 ± 122	$P < 0.02$
Rats	10	Acacia solution	1-10	719 ± 33	
Rats	10	400 mg./Kg. Meprobamate	1-10	997 ± 62	$P < 0.001$
Rats	10	Acacia solution	1-10	719 ± 33	
Rats	8	200 mg./Kg. Meprobamate	1-10	830 ± 37	$P < 0.05$
Rats	8	Acacia solution	1-10	743 ± 9	
Rats	8	500 mg./Kg. Meprobamate post-toxin	1-10	1227 ± 234	No significance
Rats	8	Acacia solution post-toxin	1-10	1103 ± 238	
		***E. coli* Endotoxin**			
Mice	10	533 mg./Kg. Meprobamate	1-50	865 ± 81	$P < 0.02$
Mice	10	Acacia solution	1-50	622 ± 38	
		***S. typhosa* Endoxotin**			
Mice	10	533 mg./Kg. Meprobamate	1-50	861 ± 79	$P < 0.02$
Mice	10	Acacia solution	1-50	624 ± 35	

ports the current concept of the nonspecific nature of such lipopolysaccharide extracts from Gram-negative bacilli.

Bacterial Studies.—It has been previously shown (4) that reserpine is capable of significantly prolonging the life of animals injected with an overwhelmingly lethal dose of *Diplococcus pneumoniae*. Experiments with meprobamate and *D. pneumoniae* did not yield conclusive results although almost a thousand animals were used in an effort to establish a consistent response.

With our bacterial inoculum, infected animals always died between the sixteenth and forty-eighth hours postinjection. A single dose of 420 mg./Kg. meprobamate either one hour before or one hour after *D. pneumoniae* challenge did not affect survival. Preconditioning the animals with four injections of 300 mg./Kg. meprobamate eight hours apart prior to bacterial inoculation had no effect upon survival, and injections of the drug on the same schedule commencing immediately after bacterial inoculation tended to shorten survival. A single injection of 720 mg./Kg. meprobamate one hour prior to bacterial introduction resulted in increased survivals, while a dose of 800 mg./Kg. reduced survivals as compared to controls. In neither case, however, was statistical significance achieved.

During our experiments, we noted with interest the report by Grosz and Norton (5) that chlorpromazine increased the susceptibility of mice to infection with *Salmonella enteritidis*. Therefore, we compared the relative intensity of this response in both chlorpromazine and meprobamate-treated animals inoculated with *S. enteritidis*. From the results plotted in Fig. 1 it is evident that chlorpromazine did significantly shorten the life of infected animals in the dose employed. Meprobamate also reduced the survival time of infected animals, but to a far lesser extent than did chlorpromazine.

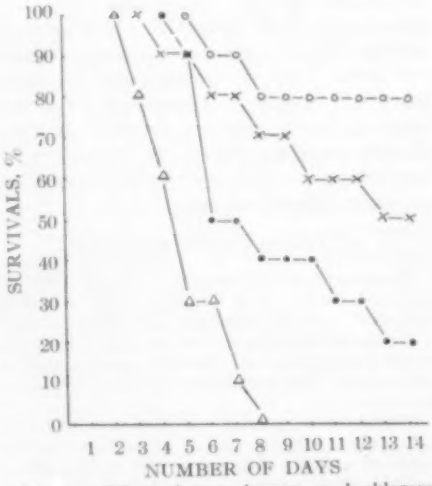

Fig. 1.—Effect of meprobamate and chlorpromazine on survival time of mice infected with *Salmonella enteritidis*. ○ Acacia, meprobamate control; X water, chlorpromazine control; ● meprobamate, 533 mg./Kg.; Δ chlorpromazine, 2 mg./Kg.

Similar comparisons were then undertaken using *Salmonella typhimurium* as the stress organism. In several trials, no significant variations from control values were demonstrated either by chlorpromazine or meprobamate treatment. From these results, we conclude that the enhancement of lethality by chlorpromazine noted by Grosz and Norton, and the enhancement of lethality by both chlorpromazine and meprobamate noted by us is not a universal response to bacterial stress.

SUMMARY AND CONCLUSIONS

1. Mice and rats were subjected to overwhelming bacterial stress utilizing purified exotoxins, lipopolysaccharide endotoxins, and broth cultures.

2. Pretreatment with meprobamate exerted a significant life-prolonging effect in *Clostridium tetani* and *Clostridium botulinum* exotoxemias in both mice and rats. This response was correlated to the dose of the drug employed.

3. Pretreatment with meprobamate resulted in a significant prolongation of life in mice inoculated with lethal doses of *E. coli* and *S. typhosa* endotoxins.

4. Various dosage schedules of meprobamate were evauated in overwhelming *D. pneumoniae* infection. No significant effects on survival were demonstrated.

5. Meprobamate enhanced the lethality of *S. enteritidis* in mice, but to a lesser extent than chlorpromazine. Neither meprobamate nor chlorpromazine significantly altered the lethality of *S. typhimurium* for mice.

6. In laboratory animals, the antistress spectrum of meprobamate extends beyond emotional stress into the physiological stress of bacterial toxemias.

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Investigation of *Machilus macrantha* Nees. I*

Pharmacognostical and Phytochemical Study of Root

By K. N. GAIND and S. K. BAVEJA

The root of *Machilus macrantha* Nees. has been studied for physical characteristics, histology, extractives, ash, and alkaloidal constituents. Anatomical characteristics include: a continuous band of small stone cells constituting phelloderm; a pericycle consisting of large stone cells; isolated groups of stone cells in the phloem; lignified phloem and xylem fibers with narrow and large lumen, respectively; copious mucilaginous matter in the phloem parenchyma cell walls; and oil cells in cortex, phloem, and xylem. The extractives showed the presence of phytosterols, glycosides, and volatile oil. From the limed drug, extraction and isolation of a base as hydrochloride, provisionally named "machiline hydrochloride," melting with decomposition between 248–250°, two alkaloids as picrates and one reineckate are described. The yield of machiline hydrochloride was highest (0.0725 per cent) from the root collected in the month of May. Paper chromatography of the extract revealed the presence of at least five alkaloids.

THIS STUDY was prompted by the information that the root of *Machilus macrantha* Nees. is considered useful in hypertension by the practitioners of the Indian indigenous system of medicine. It has been reported in the literature (1-3) that the bark of this plant is used in consumption, asthma, and rheumatism, and its leaves are applied to ulcers.

No prior investigation of this plant has been reported. From another species of this genus, *M. thunbergii*, an oil was extracted and its constants and constituents were studied (4).

This study was made to provide sufficient pharmacognostical characterization for the identification of *M. macrantha* root and to establish a scientific basis for its use in hypertension.

* Received September 10, 1959, from the department of Pharmacy, Panjab University, Chandigarh, India.
The authors are sincerely grateful to Mr. R. S. Sandhu, Pharmacognocist, Medical College, Amritsar, for his assistance in pharmacognostical study.

EXPERIMENTAL

Materials Used.—The roots and flowering shoots of *M. macrantha* were obtained from Kulathupuzha, Kerala State (India), through the courtesy of Mr. T. K. Devakaran, forest range officer. It was authenticated by Mr. B. M. Raizada, Head, Division of Forest Botany, Forest Research Institute, Dehradun. The dried roots, pulverized to moderately coarse powder, were used for phytochemical work.

The Plant.—*Machilus macrantha* Nees. of the *Lauraceae* family known as Gumara or Gumbara in Konkan; Uravu in Malayalum; Anaikkuru, Kollamavu, and Mullai in Tamil; and Ululu in Sinhalese, has been described previously (1, 5). It is a large tree attaining a height of 20–25 M. and is fairly abundant from Konkan southwards in the Western Peninsula (India) and Ceylon.

Physical Characteristics of Root.—The roots examined (Fig. 1) were large pieces, 15–20 cm. in diameter, and small pieces, 3–3.5 cm. in diameter. They were cylindrical, rarely flattened, and light in weight when dry. Externally, they were yellowish-brown to dirty buff colored with longitudinal wrinkles. Some of the large pieces showed transverse cracks. In small pieces the transverse cracks were rare but transverse ridges and occasionally lenticels were present. They also showed rootlet scars. Internally, there was a narrow, granular, brown to dark brown bark, 3–3.5 mm. thick in small pieces and 7–10 mm. thick in large pieces, and an inner pale brown to reddish-brown wood with no growth rings. The fracture of the bark was short, smooth toward the periphery and granular toward the inside; that of the wood was splintery. The dried root had a slight aromatic odor and a slimy taste. The bark, on soaking in water, ejected copious mucilaginous matter.

Fig. 1.—Root of *Machilus macrantha* Nees.

Histology of *M. macrantha* Nees. Root.—Roots of 3–3.5 cm. diameter were selected (see Figs. 2 and 3). Samples from these were prepared by the usual pharmacognostic methods (6). Sections were cut on a wood microtome and by free hand sectioning. Numerous temporary mounts and permanent mounts of transverse, radial longitudinal, and tangential longitudinal sections of the root specimens were made and examined microscopically. For study of individual cells, different tissues were macerated in potassium chlorate and nitric acid reagent (7) and slides

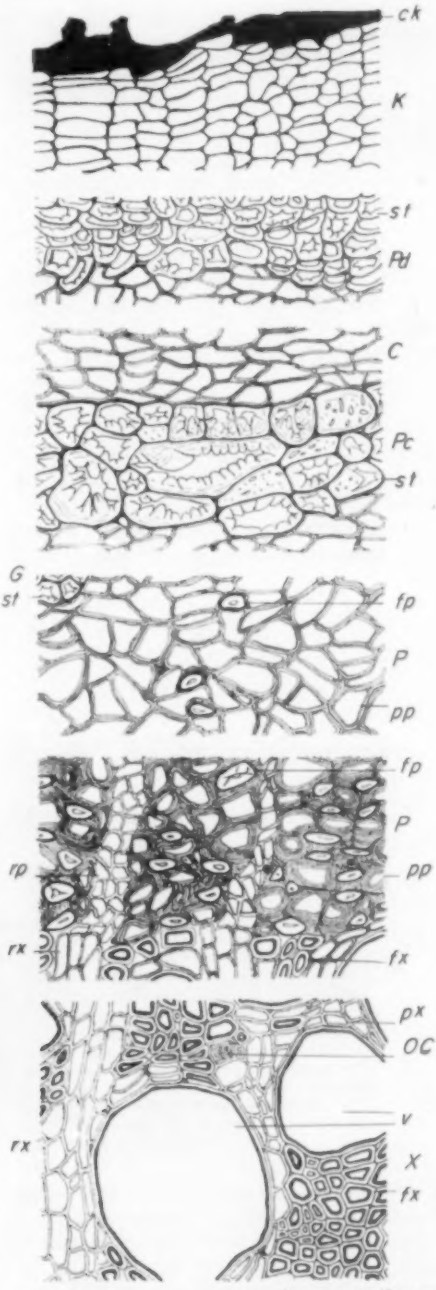

Fig. 2.—Transverse sections of representative portions of *Machilus macrantha* Nees. root. *K*, cork; *Pd*, phelloderm; *C*, cortex; *Pc* pericycle; *P*, phloem; *x*, xylem; *ck*, compressed cork cells; *st*, stone cells; *fp*, phloem fibers; *pp*, phloem parenchyma; *rp*, phloem rays; *px*, xylem parenchyma; *oc*, oil cells; *v*, xylem vessels; *G. st*, group of stone cells; *fx*, xylem fiber; *rx*, xylem ray. ×140.

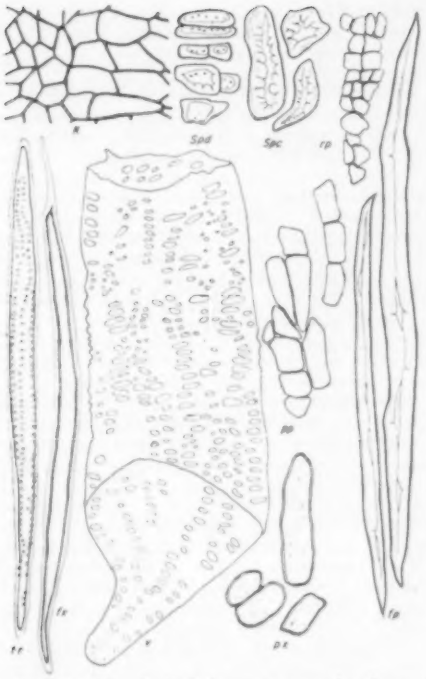

Fig. 3.—Histological elements occurring in macerated tissues and powdered root of *Machilus macrantha* Nees. *K*, cork cells in surface view; *spd*, stone cells of phelloderm; *spc*, stone cells of pericycle; *rp*, phloem ray cells; *pp*, phloem parenchyma; *fp*, phloem fibers; *px*, xylem parenchyma; *v*, vessel with large and small pits; *fx*, xylem fiber; *tr*, tracheid. ×100.

mounted in 50% glycerin. In the following description, the symbols *R*, *T*, and *L* refer to measurements made in radial, tangential, and longitudinal directions, respectively.

In a cross section 33 mm. in diameter, the bark occupied 7 mm. and the wood 26 mm. The cork zone was up to 25 layers of cells deep, covered on the outside by a few layers of collapsed cork cells. The cells were rectangular to somewhat squarish, arranged in more or less radial files; polygonal in tangential section; *R*, up to 2.8 μ; *T*, 11.0 μ; and *L*, 8.0 μ; with suberized walls. The phelloderm was composed of up to eight layers of somewhat rectangular to isodiametric stone cells, 1.5 to 12.0 μ, arranged in radial rows corresponding to those of cork cells; cell walls thickened, usually along radial and interior tangential sides, yellow, lignified and traversed by a few simple pore canals; lumen comparatively large but variable and occluded with dark reddish-brown contents. The cortex parenchyma cell walls were somewhat thickened with mucilaginous matter. Reddish-brown contents and a few simple starch grains were present in some cells. A few yellow oil cells were interspersed in the cortex, phloem, and xylem parenchyma. The conspicuous pericycle was composed of three to five layers of stone cells of 5 to 25 μ; cell walls thick, yellow, lignified with a few striations in some; traversed by funnel-shaped simple and branched pore canals; lumen narrow but variable, totally occluded in a few, and devoid of cell contents. The phloem comprised 75% of the thickness of the bark. The phloem rays traversed the inner two-thirds of the phloem, were usually up to three cells wide, rarely four, 15 to 25 cells deep longitudinally, somewhat wavy, tapering outwards, and in continuation with their counterparts in the xylem. The phloem parenchyma cell walls were thickened with mucilaginous matter, more so on cells lying interiorly, the thickening being reddish brown and striated. The phloem fibers were numerous, thicker and longer in the interior region, up to 1,240 μ long and 60 μ thick, with yellow lignified walls traversed by a few simple rarely branched pits; lumen narrow, partially obliterated; ends bluntly pointed, rarely sharp. A few small groups of yellow lignified stone cells with narrow lumen were also present. The primary xylem was represented by a group of xylem elements in the center. The secondary xylem had xylem rays of straight to curved character up to four cells wide and 20 cells deep longitudinally, with irregularly rectangular cells up to 50 μ long. The rounded or somewhat oblong vessels occurred singly or in groups of two to four, with their adjacent walls flattened. They were up to 860 μ long and 300 μ broad. The vessel walls bore numerous pits of varying size, the larger ones being transversely or obliquely elongated. The wood parenchyma was scarce, the cells being usually elongated longitudinally; *L*, up to 250 μ; *R* and *T*, 50 μ. A few tracheids up to 1,100 μ long and 50 μ broad with pitted walls were also present. The rest of the xylem was constituted of fibers having moderately thick, yellow, lignified walls and a relatively large lumen. They measured up to 1,250 μ long and 50 μ broad and were arranged together with xylem parenchyma and tracheids in more or less radial files. Intercellular spaces were present.

Powdered M. macrantha Root.—This was pale brown with a slimy taste and aromatic odor. Under the microscope it showed: numerous fragments of wood fibers with thick, yellow, lignified walls and comparatively large lumens; phloem fibers with narrow and at places obliterated lumens and thick, yellow, lignified walls bearing simple pits; stone cells isodiametric, elongated or irregularly shaped, single or in groups with lumens of variable size, and thick, yellow, lignified walls bearing simple and branched pits; fragments of vessels and tracheids with pitted walls; scattered fragments of cork, with polygonal cells having suberized walls; and a few simple starch grains 0.5–1.0 μ, conspicuous in Smith's starch reagent.

Extractives.—Root powder (15 Gm.) was extracted successively with solvents in the order given below, employing a Soxhlet extraction apparatus, the preceding solvent being completely removed from the marc before starting extraction with the next solvent. The percentages of the extractives obtained were: petroleum ether (40–60°), 2.38%; ether, 0.32%; chloroform, 0.27%; and absolute alcohol, 3.90%. Volatile oil was detected in petroleum ether, phytosterols in petroleum ether and ether, and glycosides in petroleum ether, ether, and chloroform extracts. Dilute hydrochloric acid

extract of root powder gave positive tests with alkaloidal reagents.

Determination and Analysis of Ash.—The methods used for determination of ash content were those of the British Pharmacopoeia 1958 (8). The results were as follows: total ash, 2.3%; water-insoluble ash, 1.78%; water-soluble ash (by difference), 0.52%; acid-insoluble ash, 0.235%; and acid-soluble ash (by difference), 1.545%. The total ash contained sodium, calcium, magnesium, nitrate, and carbonate.

Extraction and Isolation of Machiline Hydrochloride.—Root powder in 0.8-Kg. quantities was mixed with 10% of its weight of calcium hydroxide, moistened, allowed to stand for four hours, and dried at 60°. The limed drug was extracted with alcohol (95%) in a Soxhlet apparatus until exhausted of alkaloids. The alcohol was recovered from the extract and the residue kneaded with 2% hydrochloric acid in portions until the alkaloids were completely taken up in acid solution. The pH of the solution was adjusted to 4.0 with dilute ammonia solution and extracted with chloroform until the latter extracted no more alkaloids. The pH was then raised to 8.0 and extraction with chloroform was repeated. The aqueous solution was then made highly alkaline with 28% ammonia solution, extracted with chloroform, the chloroform solution dried over exsiccated sodium sulfate, the chloroform recovered, the residue taken up in dry ether, and dry hydrochloric acid gas bubbled through the ether solution. The precipitated alkaloidal mass, on repeated crystallization from absolute alcohol, gave white crystals, elongated rectangular plates (under the microscope), melting range 248–250° (decomposition). It did not give a positive test with the usual precipitating alkaloidal reagents. It has been provisionally named "machiline hydrochloride." The yield of machiline hydrochloride obtained from *M. macrantha* root, collected in different months was as follows: February, 0.056%; May, 0.0725%; August, 0.038%; and November, 0.031%. The bark of the root collected in August was separated. It constituted 21.5% by weight, of the whole root and on extraction yielded 0.0045% of machiline hydrochloride; the wood yielded 0.0472%. Studies on the chemical constitution and pharmacological action of machiline hydrochloride are reported in Part II of this series.

Isolation of Two Alkaloids as Picrates.—From the mother liquor left after crystallization of machiline hydrochloride, the solvent was recovered and the alkaloids precipitated with picric acid in aqueous solution. The precipitated picrates were separated, dried in a vacuum desiccator, taken up in absolute alcohol, and chromatographed on a column of alumina. Of the five bands formed, the lowermost yellowish-brown band (fraction 1) and the next higher yellow band (fraction 2) were eluted with absolute alcohol, the eluted fractions concentrated and kept separately in desiccators containing, respectively, ether and benzene. From fraction 1, a microcrystalline brown precipitate melting with decomposition between 157–162° was obtained during one week, and from fraction 2, yellow crystals in the form of elongated rectangular plates, melting with decomposition between 270–272°, were obtained in three to five days.

Isolation of the Water-Soluble Alkaloid as a Reineckate.—The highly alkaline aqueous liquid left after extraction of the chloroform-soluble alkaloids still gave a strong test for alkaloids. It was concentrated under reduced pressure, the separated ammonium chloride was filtered off, and the alkaloid precipitated by addition of 2% solution of ammonium reineckate in dilute hydrochloric acid. The precipitated complex was separated, dried, taken up in dry acetone, and passed through a column of alumina. The clear, bright orange-red eluate was concentrated and kept in a vacuum desiccator overnight. The orange-red crystals formed were separated, washed with ether, and dried. The compound melted with decomposition at 260–262°.

Paper Chromatographic Analysis.—The ascending method was employed. The apparatus consisted of a specimen jar, 40 cm. × 10 cm., fitted with a rubber stopper through which passed a glass rod bearing hooks at the lower end. Paper strips (30 cm. × 2 cm.) were cut from Whatman No. 1 filter paper and buffered at pH 4.0 by dipping in 5% aqueous solution of monosodium citrate, blotting, and drying at 60° for twenty-five minutes. The solvent system consisted of *n*-butanol:water:citric acid, 50:50:1 Gm.; the organic layer was employed as the mobile phase. Fresh solvent was necessary for each resolution to achieve reproducible R_f values. Samples for analysis consisted of the concentrated alcoholic extract and the residues of the chloroformic and aqueous fractions derived from it, redissolved in alcohol. Development was performed at 15° ± 2°. The chromatograms were air dried and dipped in Munier's modified Dragendorff's reagent (9), and R_f values were calculated. In all, 5 bands were formed on the different chromatograms, their average R_f values being 0.90, 0.71, 0.47, 0.25, and 0.08. The water-soluble alkaloid formed the lowermost band which gave a purple spot with the Munier's reagent while the others gave orange spots on yellow background. The two high R_f values spots were pale yellow to yellowish brown even before treatment with the modified Dragendorff's reagent.

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Investigation of *Machilus macrantha* Nees. II*

Pharmacological Action and Chemical Constitution of Machiline

By K. N. GAIND and S. K. BAVEJA

Machiline, isolated as the hydrochloride from the root of *Machilus macrantha* Nees., has been studied for its pharmacological action and chemical characteristics. It has been found to lower blood pressure of experimental animals by a direct depressant effect on the myocardium. It has no antibacterial activity. A number of derivatives have been prepared. Based on the results of (*a*) microanalysis for elements of the base, its hydrochloride, picrate, acetyl, and benzoyl derivatives, (*b*) chemical tests for functional groups, (*c*) microestimation of —OCH_3 in the base and —NCH_3 in the methiodide, and (*d*) infrared and ultraviolet absorption spectra, two structures are proposed for machiline

IN THE PREVIOUS report (1), the isolation of a base "machiline" as a hydrochloride from the root of *Machilus macrantha* Nees. was reported. The present investigation was undertaken to study the pharmacological action and chemical constitution of this base.

METHODS AND RESULTS

Pharmacological Actions.—Before undertaking chemical characterization, the isolated hydrochloride was studied for its effect on the cardiovascular system and on smooth muscle and for its antibacterial activity.

Cardiovascular System.—Arterial blood pressure was recorded from the common carotid artery in dogs weighing 7–9 Kg., anesthetized with pentobarbital sodium (32 mg./Kg.) intravenously. The dose of machiline hydrochloride solution was injected through the cannulated femoral vein. With a dose of 0.1 to 1.0 mg., there was a fall in blood pressure (2 to 11 mm.) proportionate to the quantity administered. With larger doses the fall was only slightly greater, the maximum for a dose of 50 mg. being 18 mm.

Machiline hydrochloride (10 mg.) caused a decrease in the amplitude of contraction of the intact heart in an anesthetized dog with open chest. On the isolated heart (Langendorff's method) (2) of rabbits weighing 1–1.5 Kg., a dose of 0.1 mg. caused slowing and irregular rhythm and a dose of 1.0 mg almost stopped the heart by slowing and gradual decrease in the amplitude of contraction. Subsequently the heart recovered gradually in about ten minutes. In the intact heart of a pithed frog, a dose of 0.1 mg. caused varying grades of heart block, and a dose of 1.0 mg. caused arrest in diastole. Subsequently the heart recovered completely in about five minutes through gradually diminishing grades of heart block.

In perfusion experiments, with 0.1 to 1.0 mg. of machiline hydrochloride the flow of frog Ringer's fluid through the peripheral vessels of a frog increased for a few minutes, while the flow of Locke's solution through the coronary vessels of an isolated rabbit's heart decreased for several minutes, but the magnitude of the effects was slight and insignificant. The kidney volume and blood pressure of an anesthetized dog showed simultaneous fall with doses of 1 to 15 mg. and this effect was not blocked by previous injection of atropine. These observations suggest that the hypotensive effect of machiline is due to its direct depressant effect on the myocardium.

Action on Smooth Muscle.—A dose of 1.0 mg. of machiline hydrochloride had a depressant effect on the strips of intestine (Magnus' method) (3) of rabbits weighing 1.0–1.5 Kg., in an isolated organ bath with inner bath of 25 ml. capacity.

Antibacterial Activity.—In concentrations up to 1 in 500, machiline hydrochloride failed to inhibit the growth of *Staphylococcus aureus*, *Bacillus subtilis*, *Escherichia coli*, and *Salmonella typhi* in nutrient broth and of *Mycobacterium tuberculosis* var. *hominis* in the medium suggested by Isao Yamane (4).

Chemical Characterization of Machiline.—

Anal.—Calcd. for $C_{16}H_{17}NO_3.HCl$: C, 62.41; H, 5.90; N, 4.55; Cl, 11.58. Found: C, 61.46; H, 6.32; N, 4.35; Cl, 11.13.

The picrate from 50 mg. of the hydrochloride was precipitated with picric acid in aqueous solution and crystallized from water as yellow crystals (51 mg.) in the form of elongated rectangular plates, melting with decomposition between 205–207°.

Anal.—Calcd. for $C_{16}H_{17}NO_3.C_6H_2(OH)(NO_2)_3$: C, 52.78; H, 4.03; N, 11.20; Mol. wt., 500.2. Found: C, 53.61; H, 4.40; N, 11.47; Mol. wt., 465 (Rast method).

The base from 100 mg. of the hydrochloride was precipitated with ammonia in aqueous solution and crystallized from 50% alcohol as a microcrystalline white powder (60 mg.) melting with decomposition at 210–211°.

Anal.—Calcd. for $C_{16}H_{17}NO_3$: C, 70.81; H, 6.32; N, 5.16; Mol. wt., 271.2. Found: C, 70.29; H, 6.60; N, 4.74; Mol. wt., 301 (Rast method).

Machiline hydrochloride did not respond to tests with alkaloidal reagents as has already been reported (1). The murexide test for purines was also negative. Heating with dilute as well as concentrated hydrochloric acid did not split the molecule of machiline. It decolorized bromine in aqueous, alcoholic, and glacial acetic acid solutions, and de-

* Received September 10, 1959, from the Department of Pharmacy, Panjab University, Chandigarh, India.

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colorized potassium permanganate in aqueous solution. It did not reduce Fehling's solution, but a silver mirror was formed with ammoniacal silver nitrate. It gave a negative test with Schiff's reagent and also with 2,4-dinitrophenylhydrazine reagent (5). The base neither gave effervescence with, nor dissolved in sodium bicarbonate solution. It dissolved in caustic soda solution and also in ammonia solution, but gave no color with ferric chloride in aqueous or alcoholic solutions. It did not give a green color with gallic acid reagent (6) or a red color with the phloroglucinol-sulfuric acid reagent of Gaebel (7).

Anal.—Calcd. for one methoxy in $C_{16}H_{17}NO_3$: $-OCH_3$, 11.3; Found: $-OCH_3$, 7.5.

On heating the base with soda lime, vapors having an ammoniacal odor were evolved and when these were passed into a solution of hydrochloric acid gas in ether a precipitate was formed, which was identified as ammonium chloride. On treating the base with ice-cold nitrous acid, a gas was evolved and a yellow solid precipitated. The latter gave a negative Liebermann test. When the yellow supernatant liquid was made alkaline, no solid separated out. Rimni's (5) and the carbon disulfide reagent (5) tests were also negative.

Acetylmachiline.—Machiline hydrochloride (100 mg.), acetic anhydride (1.5 ml.), and pyridine (5 drops) were boiled together under reflux on a sand bath for one hour, cooled, poured into water (80 ml.), set aside overnight, and the precipitate formed was crystallized from 50% alcohol. A crop of white crystals in the form of elongated rectangular plates, m. p. 197–199°, was obtained.

Anal.—Calcd. for $C_{16}H_{15}NO_3.(CH_2CO)_2$: C, 67.57; H, 5.96; N, 3.94. Found: C, 66.70; H, 6.00; N, 3.73.

Benzoylmachiline.—Benzoylation was effected by the Schotten-Baumann process and the product crystallized from absolute alcohol as elongated rectangular plates, m. p. 214–216°.

Anal.—Calcd. for $C_{16}H_{15}NO_3(C_6H_5CO)_2$: C, 75.12 H, 5.26; N, 2.92. Found: C, 76.20; H, 5.54; N, 2.88.

Machiline Methiodide.—Machiline (100 mg.) in methanol (5 ml.) and methyl iodide (0.5 ml.) were warmed together under reflux for one hour. The volatile components of the reaction mixture were volatilized off and the residue dried to constant weight at 120° (154 mg.) was crystallized from dry acetone in a desiccator containing ether. The crystallized compound, after drying at 120°, melted between 184–187°.

Anal.—Calcd. for $C_{16}H_{17}NO_3.CH_3I$: $-CH_3$ (attached to N), 3.63. Found: $-CH_3$ (attached to N), 3.51.

Infrared Spectrum.—The infrared spectrum of machiline (Fig. 1) was taken in a Nujol mull on a single beam Perkin-Elmer spectrophotometer making necessary corrections for interferences due to atmospheric water and carbon dioxide.[1] The brief analysis (Table I) of the infrared record indicates the groups responsible for some of the absorption bands; the assignments were done on an empirical basis.

Ultraviolet Spectra.—The ultraviolet spectra (Fig. 2) were taken on a Hilger Uvispeck photoelectric spectrophotometer equipped with a fused silica prism.[2] Both machiline and its hydrochloride showed approximately the same absorption maxima in the ultraviolet region and no absorption in the visible region of light. The base showed absorption maxima at 226 and 285 $m\mu$ in absolute alcohol; its hydrochloride at 200, 224, and 282 $m\mu$; and there was no inflexion near 315 $m\mu$ even in a more concentrated solution. The maxima were comparable to those of naphthalene and quinoline except for the lack of inflexion. The inflexion could be absent if (*a*) a quinoline structure were present as its tetrahydro form or (*b*) a naphthalene structure were present as 1-phenylnaphthalene (or 2′-hydroxy-1-phenylnaphthalene or 2′-methoxy-1-phenylnaphthalene, etc.). The latter structure would make it rather difficult to fit in nitrogen present as an amine group which in such cases would shift the maxima too much in the acidic condition, whereas not much shifting was observed in the curve of base hydro-

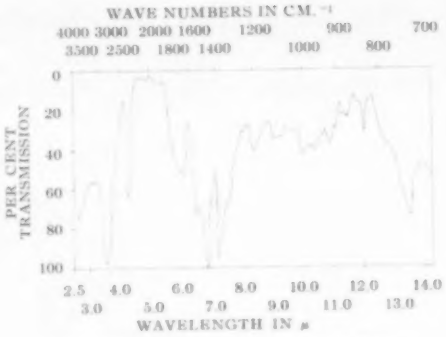


Fig. 1.—Infrared spectrum of machiline.

TABLE I.—EMPIRICAL ASSIGNMENT OF GROUPS RESPONSIBLE FOR ABSORPTION BANDS IN THE INFRARED SPECTRUM OF MACHILINE

Absorption Band Peak, cm.⁻¹	Assigned Grouping
3175	CH_2
2381, 2105, 2000, 1835	≡N (these should be weak bands but were found as strong bands due to faulty compensation)
1600	C_6H_6, C=C, C= N in N heterocyclic (?)
1504	C_6H_6 substituted, C_5H_5N (?)
1399	C_6H_5OH, —C—CH_2 (?)
1333	C_6H_5—N=, C_6H_5OH, —OH
1250	C_6H_5OR
1212, 1093, 1058, 1020, 976	1:2, 1:4, 1:2:4 substituted C_6H_6
813	N (heterocyclic), $(CH_3)_2C$ (?), C—CH— (?)
719	N (heterocyclic)

[1] The spectrum was obtained through the courtesy of Dr. M. L. Dhar of the Central Drug Research Institute, Lucknow.

[2] The spectra were prepared in the laboratory of the Royal Institute of Technology, Glasgow, through the courtesy of Dr. H. C. Mital.

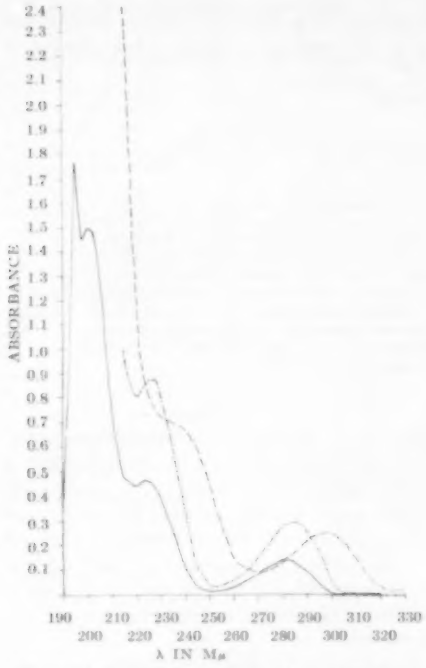
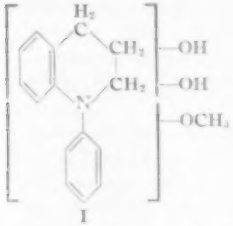


Fig. 2.—Ultraviolet spectra of machiline.——machiline hydrochloride in water 0.0009818%; – – – – machiline hydrochloride in decinormal sodium hydroxide 0.0009818%; –·–·–·–machiline base in ethanol 0.0014374%.

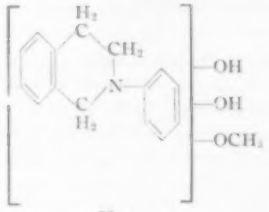
chloride. Taking the quinoline structure, its tetrahydro form would not show marked shift in maxima and is in agreement with the observed maxima of base hydrochloride. In alkaline solution the spectrum of machiline hydrochloride was markedly influenced (by the alkali) above 230 mμ, indicating that nitrogen function is near a benzene ring, i.e., there is a quinoline rather than an isoquinoline system or else a phenyl group is attached to nitrogen in the isoquinoline form.

DISCUSSION

The microanalytical data of the base and its derivatives show that the molecular formula of machiline is $C_{19}H_{17}NO_3$ and it has two OH groups and one OCH_3 group. The solubility of the base in alkali hydroxides and the infrared spectrum indicate that at least one of the OH groups is phenolic though it may be in a somewhat hindered position. It does not give the characteristic test for phenols with ferric chloride solution but many other phenolic and enolic compounds behave similarly (7) and, therefore, phenolic OH cannot be considered absent. Chemical tests, infared spectrum, formation of a quaternary ammonium compound with methyl iodide accompanied with increase in weight of 100 mg. of the base to 154 mg. of the methiodide, and microanalytical data for $-CH_3$ (attached to N) in the methiodide, all point to the tertiary nature of the nitrogen function. Further the infrared spectrum indicates the nitrogen function to be in a heterocyclic system. The ultraviolet spectra corroborate this finding; specifically, there is a tetrahydroquinoline (or tetrahydroisoquinoline with a phenyl group attached to N) structure. This, together with OCH_3 and OH groups, accounts for $C_{19}H_{17}NO_3$ leaving C_6H_5 which evidently constitutes a phenyl group and must be attached to a nitrogen function to account for the tertiary nature of the latter in the tetrahydroquinoline (or tetrahydroisoquinoline) nucleus. This conclusion is further strengthened by the maxima at 1,333 cm.$^{-1}$ of the infrared spectrum. On this basis the likely structures for machiline are those represented by I and II.



I



II

The investigated alkaloids of laurels are known (8) to possess an aporphine ring structure, which has tetrahydroisoquinoline nucleus and on this analogy, structure II may be considered more likely for machiline.

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The Selective Determination of Isopropamide Iodide, A Low-Molecular Weight Quaternary Ammonium Compound*

by RALPH S. SANTORO

Available indicator extraction methods were inadequate for the determination of isopropamide iodide in the presence of amine bases. The substance was complexed with methyl orange in pH 10.2 buffer and extracted into chloroform. Re-extraction of the color into 1 *N* hydrochloric acid and its spectrophotometric determination gave quantitative results. Calibration curves for other short chain quaternary amines were determined and compared to a known solution of methyl orange. Agreement with the theoretical value was obtained down to tetra-*n*-propyl ammonium iodide. Application of the method to the determination of isopropamide iodide in a pharmaceutical preparation gave results ranging from 98.7 to 101.3 per cent of the theoretical amount present.

IN AN INVESTIGATION to determine a low-molecular weight quaternary amine, isopropamide iodide,[1] in the presence of amine bases, it became evident that existing indicator extraction methods were inadequate because of poor reproducibility and sensitivity.

Analytical procedures for quaternary ammonium compounds are well represented in the literature. Auerbach (1, 2) determined cetyl pyridinium chloride and other high-molecular weight quaternary amines using bromophenol blue. Cochin and Woods (3) assayed tetraethylammonium with bromocresol purple, extracting the complex from pH 7.0 buffer with chloroform. Ballard, Isaacs, and Scott (4) attempted to elucidate the principles of complex extraction of amines and determined the calibration curves for large chain quaternary compounds using bromothymol blue. They were unsuccessful in determining low-molecular weight quaternaries due to poor complexing and extraction. Other workers have determined large chain surfactants by indicator displacement methods (5, 6, 7).

Similar methods for amine bases are also well documented, usually differing from quaternary methods by the pH at which the complex is extracted. Brodie and Udenfriend (8) determined cinchona alkaloids with methyl orange in acid medium and indicated its application to other amine bases. Keller and Ellenbogen (9) adapted this method for the determination of *d*-amphetamine. Adaptions using different indicators, pH conditions, and solvents have also been described (10–13).

It was found that bromophenol blue, bromothymol blue, and bromocresol purple extractions were incomplete, with a corresponding lack of reproducibility in the results. Alterations in pH and the use of different solvents did not improve the extraction or the reproducibility. Below pH 7.0, the amines complexed and interfered with the determination. No reaction was shown in the direct photometric methods by indicator displacement.

This paper describes an indicator extraction method to determine isopropamide iodide selectively and its use in the determination of certain other low-molecular weight quaternary amines.

EXPERIMENTAL

Reagents.—*Chloroform*, reagent grade. *Hydrochloric acid solutions*, 1.0 *N* and 0.1 *N*. *Buffer solution pH 10.2*, 87 Gm. of dipotassium hydrogen phosphate, reagent anhydrous, and 42 Gm. of sodium carbonate, reagent anhydrous, were dissolved in 2 L. of distilled water. *Methyl orange buffer solution*, 1 L. of 10.2 buffer solution was saturated with methyl orange, reagent grade, by adding 2 Gm. and shaking vigorously. The solution was filtered through Whatman No. 1 filter paper and extracted with chloroform until the chloroform layer was colorless. *Stock solutions of quaternary ammonium salts*, 50 mg. of salt was dissolved in 500 ml. of distilled water. This solution was diluted 20 to 100 ml. with 0.1 *N* hydrocholoric acid. A final concentration of 0.020 mg./ml. of the salt was obtained. *Stock solutions of amine salts*, 10 mg. of each substance was dissolved in 100 ml. of distilled water. The concentration of amine salt was 0.100 mg./ml.

Apparatus.—*Klett-Summerson photoelectric photometer with micro tubes.*

Preparation of Calibration Curve.—Into four 250-ml. separatory funnels, 20 ml. of methyl orange buffer solution was added. Into the first three separatory funnels, 1, 2, and 3 ml., respectively, of the final quaternary ammonium salt solution were pipetted. The fourth separatory funnel served as the reagent blank. The solutions were extracted

* Received August 29, 1958, from Smith Kline and French Laboratories, Philadelphia 1, Pa.

[1] The material used in this study was Darbid, Smith Kline and French brand of (3-carbamoyl-3,3-diphenylpropyl)-diisopropylmethyl ammonium iodide.

with four 25-ml. portions of chloroform. The filtrates were collected in 250-ml. glass-stoppered Erlenmeyer flasks through a pledget of cotton previously wet with chloroform. After the last extraction, the cotton was washed with a 25-ml. portion of chloroform.

Into each flask was pipetted 10 ml. of 1 *N* hydrochloric acid. The flasks were stoppered and shaken vigorously for about one minute. After the layers separated, at least 2 ml. of the clear supernatant aqueous phase was decanted into a micro-Klett tube. The absorbance was read on a Klett-Summerson photoelectric photometer using a 52 filter. All readings were made against 1 *N* hydrochloric acid. The reagent blank was subtracted from the standard readings and each standard weight in mg. was divided by its reading. The above standard weights were 0.020, 0.040, and 0.060 mg. of the quaternary salt, in that order. The factors obtained were averaged.

Determination.—Isopropamide iodide was assayed in the presence of amine bases. The determination was the same as the calibration except that to 0.040 mg. (2 ml.) of the standard isopropamide iodide solution 0.200 mg. (2 ml.) of the amine solution was added. The recovery was calculated as follows:

$$\frac{\text{photometer reading} \times \text{average factor}}{\text{mg. isopropamide iodide added}} \times 100 = \% \text{ recovered}$$

APPLICATIONS

This method was applied to the determination of isopropamide iodide in the presence of prochlorperazine dimaleate[3] in a pharmaceutical preparation.[4] The sample was ground and shaken with 0.1 *N* hydrochloric acid. A dilution was made such that the final concentration of the quaternary salt was 0.010 mg./ml. The determination was carried out as in the calibration with 5 ml. of the final sample dilution. A sample blank was also run by extracting an additional 5-ml. aliquot from pH 10.2 buffer.

RESULTS AND DISCUSSION

Theoretical considerations and actual determinations show that the molar composition of the methyl orange complex containing one quaternary amine group is a ratio of 1:1 (14). To determine the completeness of extraction and to compare the calibration curve to a theoretical value, the ratio of the Klett readings divided by the calibration curve concentrations, in terms of the methyl orange ion, were calculated and compared to a known solution of methyl orange in 1 *N* hydrochloric acid (Table I).

Agreement with the theoretical value was obtained beginning with tetra-*n*-propyl ammonium iodide. A marked decrease in absorptivity was noted with the tetrapropyl salt at the 0.060-mg. point, which was consistent throughout the calibrations. With this compound, calibrations and determinations would have to be carried out at the 0.040-mg. concentration and below for maximum reproducibility.

No extraction of indicator complex was effected with quaternary amines having a lower molecular weight than tetra-*n*-propyl ammonium iodide. These results indicate that the groups attached to the nitrogen must be at least propyl in size, or have a combined total of twelve carbons.

The recovery of isopropamide iodide in the presence of amine bases (Table II) and from a pharmaceutical preparation containing prochlorperazine (Table III) was quantitative. The precision of the calibration curves used in the determinations is shown in Table IV. The sigma values show a deviation of 2 to 4% depending upon the concentration.

The color formed in the 1 *N* acid layer was found to be extremely stable. Samples and standards read after a two-day period did not materially change (Table V).

TABLE I.—COMPARISON OF CALIBRATION CURVE RATIOS TO METHYL ORANGE

Substance	mg./10 ml.	Ratio Klett Reading/Concn.	Methyl Orange Value, %
Methyl orange	0.030	6.811	...
Tetra-*n*-propyl ammonium iodide	0.020	6.631	97.4
	0.040	6.532	95.9
	0.060	6.023	88.4
Tetra-*n*-butyl ammonium iodide	0.020	6.615	97.1
	0.040	6.779	99.5
	0.060	6.685	98.2
Isopropamide iodide	0.020	6.816	100.1
	0.040	6.839	100.4
	0.060	6.783	99.6

TABLE II.—RECOVERY OF 0.040 MG. OF ISOPROPAMIDE IODIDE IN THE PRESENCE OF AMINE BASES

Amine, 0.200 mg.	Recovery of Isopropamide Iodide, %		
d-Amphetamine	99.3	99.6	100.3
p(2-Aminopropyl) phenol	99.6	93.3	101.3
Phenylephrine	98.4	95.6	100.8
Ephedrine	99.1	104.5	103.5
Phenoxybenzamine	104.1	100.8	98.4
Morphine	102.4	99.3	100.7
Scopolamine	103.5	97.9	104.3
Codeine	100.9	101.6	100.8

TABLE III.—RECOVERY OF ISOPROPAMIDE IODIDE FROM A PHARMACEUTICAL PREPARATION

Isopropamide Iodide, Theoretical	Prochlorperazine, Theoretical	Isopropamide Iodide, mg. Recovered	%
21.30	50.0	21.45	100.7
21.30	50.0	21.27	99.8
21.30	50.0	21.14	99.2
21.30	50.0	21.03	98.7
21.30	50.0	21.58	101.3

[3] Compazine, Smith Kline and French brand of 2-chloro-10-[3'-(N-methylpiperazinyl) propyl]-phenothiazine dimaleate.

[4] Combid Spansule capsule, SKF, a combination of isopropamide iodide and prochlorperazine dimaleate.

TABLE IV.—PRECISION OF THE CALIBRATION CURVE

Concn. 0.020 mg			Concn. 0.040 mg			Concn. 0.060 mg		
Reading	Factor $\times 10^3$	Ratio	Reading	Factor $\times 10^3$	Ratio	Reading	Factor $\times 10^3$	Ratio
83	0.241	6.551	167	0.240	6.588	251	0.239	6.602
85	0.235	6.709	160	0.250	6.312	250	0.240	6.575
87	0.230	6.867	176	0.227	6.943	260	0.231	6.839
87	0.230	6.867	176	0.227	6.943	256	0.234	6.733
88	0.227	6.946	170	0.235	6.706	261	0.230	6.865
89	0.225	7.024	179	0.223	7.061	263	0.228	6.917
87	0.230	6.867	179	0.223	7.061	255	0.235	6.707
90	0.222	7.103	177	0.226	6.982	262	0.229	6.891
82	0.244	6.472	168	0.238	6.627	256	0.234	6.733
83	0.241	6.551	175	0.229	6.903	257	0.233	6.760
89	0.225	7.024	180	0.222	7.101	266	0.266	6.996
Average								
86	0.232	6.816	173	0.231	6.839	258	0.233	6.783
Sigma								
3	0.007	0.218	6	0.009	0.253	5	0.004	0.129

TABLE V.—STABILITY OF METHYL ORANGE IN 1 *N* HYDROCHLORIC ACID

Sample No.	Original Reading	Reading After Two Days
1	104	104
2	193	194
3	280	280
4	362	364
5	98	97
6	195	188
7	276	278
8	364	362

SUMMARY

1. An indicator extraction method utilizing methyl orange at a pH of 10.2 and chloroform as solvent is described. This method allows the quantitative determination of certain low-molecular weight quaternary amines with a molecular weight equal to or greater than tetra-*n*-propyl ammonium.

2. This method is rapid, sensitive, and reproducible. Primary, secondary, tertiary amines, and alkaloids do not interfere.

3. Application of the method to the determination of isopropamide iodide in the presence of prochlorperazine dimaleate in a pharmaceutical preparation was quantitative.

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The Biological Action of Cellular Depressants and Stimulants V*

The Effect of Phenylurethane on Cellular Synthesis by *Tetrahymena pyriformis* GL

By WALTER SINGER† and JOHN J. EILER

When tetrahymena were synchronized by heat treatment in the presence of a $6 \times 10^{-4} M$ concentration of phenylurethane, the drug inhibited the increase in cell size and reduced the amount of protein synthesis normally found to occur during the period of heat treatment. Deoxyribonucleic acid (DNA) synthesis was not significantly inhibited. When this concentration of phenylurethane was added to tetrahymena cultures shortly after the end of the heat treatment, the drugged cells were not different from the controls in cell size, in protein content, or in DNA content at the onset of first synchronous division.

As a part of a general study of narcotic action we have continued our investigation into the effect of phenylurethane on parameters related to synchronous growth and cell division of *Tetrahymena pyriformis* GL. Previously we have reported that the drug delays the onset of synchronous divisions and reduces the magnitude of the maximum division indexes (1). This paper presents evidence that phenylurethane inhibits the synthetic processes giving rise to the increases in cell size and cell protein which take place during the period of temperature cycling. Deoxyribonucleic acid (DNA) synthesis does not appear to be inhibited.

METHODS

Details of culture technique, cell counting, and synchronization procedure have been given (1). Eight temperature shocks of 34° alternated with 28.2° levels were used to synchronize the cells (2). The protozoocrit method of Elliott (3) was used to measure cell volumes. Each protozoocrit was calibrated with mercury to the 0.01 mark. Five- or ten-milliliter suspensions of cells killed by the addition of 0.25 or 0.5 ml. of formalin were centrifuged at 2,500 r. p. m. for fifteen minutes, using the No. 303 metal shield in the International centrifuge No. 2 with the No. 240 head. The volume of packed cells was divided by the total cell number to obtain the volume of the average cell. Tests of the reproducibility using eight 10-ml. suspensions pipetted from the same log phase culture showed a standard error of the mean volume of about ± 1.7%.

For DNA and for some protein assays, cells were killed by holding the suspensions for thirty seconds in a 90° water bath. After centrifugation and washing with 0.24% NaCl solution, DNA was extracted by two fifteen-minute hydrolyses with 5% trichloroacetic acid (TCA) at 90°. Extraction of DNA by this procedure was found to be as complete as by the more involved Schneider method (4). Hydrolysates were combined and DNA content determined by the Dische indole method (5), as modified by Cerriotti (6) and Keck (7). Salmon sperm DNA (California Foundation for Biochemical Research) was used as the standard. The TCA insoluble residue of cellular material from the DNA-extracted tetrahymena was dissolved in 3% NaOH and assayed for protein content by the biuret method of Robinson and Hogden (8), using crystalline plasma albumin (Armour) as the standard. In some experiments, this protein assay was carried out using the TCA-insoluble material remaining after formaldehyde-killed tetrahymena had been carried through the Schneider extraction method for DNA (4).

EXPERIMENTAL AND DISCUSSION

The effect of phenylurethane was studied under two general conditions, (*a*) when the drug was added to log phase cultures just before heating the bath water up to the 34° level for the first temperature shock and (*b*) when the drug was added to synchronized cultures approximately fifteen minutes after the last (eighth) 34° level. In either case, 0.05 ml. of a stock solution of the drug in 95% ethanol was added, aseptically, to 25 ml. of culture to yield a final concentration of $6 \times 10^{-4} M$ with respect to phenylurethane. At the same time, suitable controls were provided by adding 0.05 ml. of 95% ethanol to equivalent cultures.

The experiments with the drug added before heat treatment were designed to observe the effect of phenylurethane upon the period of synchronous growth of the cell during the heat treatment and upon the subsequent first lag period. To provide basic reference points, the cell volume, DNA, protein, and cell count were always determined on samples taken from the log phase cultures immediately prior to temperature cycling. In various experiments, these same determinations were made for drugged and control cells (*a*) at about fifteen minutes after the end of the last 34° level and (*b*)

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at the onset of first synchronous division. Technical factors precluded measuring all of these parameters at both stages within any one experiment. Successful synchronization could be carried out only if the experimental cultures were maintained with a reasonably large surface-volume ratio. Accordingly, for synchronization only 25 ml. of culture was added to a 300-ml. Erlenmeyer flask. To provide sufficient cells for all the samples required, it was necessary to use at least six flasks in each experiment; three for control cells, three for drugged cells. It was then necessary to pool two of the flasks from each type of cell in order to have a sample of the size to permit the determination of cell DNA, protein, volume, and number. The remaining flask of each type of cell was used to follow the synchronous divisions. With these conditions and using a proteose-peptone and yeast extract medium, an excellent first synchronous division was always seen; the maximum division index averaged 0.73 (1). Second synchronous divisions were sometimes seen to occur, third divisions were not.

The Effects of 6×10^{-4} *M* Phenylurethane Added Before Heat Treatment.—Phenylurethane was found to modify the morphological changes observed to occur in tetrahymena during the heat treatment. After the eighth level at 34°, nondrugged tetrahymena were clearly enlarged relative to log phase cells. Most cells were distorted from the typical pyriform shape, there being an overall swelling which was particularly pronounced at the posterior two-thirds of the body. The cell apices were distinctly rounded rather than pointed. Protuberances, apparently randomly distributed about the cell body, could be seen. During the first lag period, the protuberances seemed to disappear and the cells began to look more nearly typical in shape. After the first synchronous division, the cells, though enlarged, had the general appearance of log phase cells.

The cells which were heat-treated in the presence of phenylurethane appeared enlarged at the end of the heat treatment, but were visibly smaller than the nondrugged cells at the same stage. The drugged cells were more nearly elliptical and, though somewhat swollen, showed less distortion than the controls. Cells with protuberances were rarely seen. No differences were noted in transparency or in motility of drugged cells compared to controls.

Tables I and II present data to show the extent of the increases which were found to occur in volume, protein content, and DNA content per average control and drugged cell during the heat treatment and during the first lag period. For the controls, there was an almost threefold increase in average cell volume by the end of temperature cycling (Table I). This agrees with previously reported increases in size during cycling (2, 9). When temperature-cycled in the presence of 6×10^{-4} *M* phenylurethane, tetrahymena increased in cell volume only about twofold (Table I). The average drugged cell volume was 76% (Table I) of that of the control at the end of the heat treatment.

It has been shown that tetrahymena which have been heat-treated in the presence of phenylurethane will begin first synchronous division about twenty minutes later than the control cells (1). The average size of the drugged cells at seventy-eight minutes after heat treatment was compared to that of nondrugged cells at fifty-nine minutes after (Table I). At these respective times, each suspension had just entered into the phase of first synchronous division. The average drugged cell volume was found to be only 81% of that of the control. The values for the averaged cell volumes were derived by calculation from the total packed volume of a known number of cells. It is, therefore, possible that the apparent lower volume of the average drugged cell was the result of a change in cell size distribution such that there was an increased proportion of abnormally small cells in the drugged culture. On the other hand, there could have been an overall proportional decrease in cell sizes without a notable change in distribution of sizes. It was considered important to distinguish between these two alternatives in order to permit a more adequate correlation of the volume data with the results of the study of the drug effect on the synchronous divisions. Consequently, the effect of phenylurethane on the distribution of cell sizes was investigated.

Scherbaum (9) has studied cell size distribution in normal and in synchronized cell populations. His method required calculation of cell volume from measured lengths and widths of a number of individual cells by use of the formula for volume of a prolate spheroid ($V = {}^4/_3 \pi ab^2$). Bonner and Eden (10) point out that there is no one "correct" or "logical" way of plotting data for frequency distribution of cell sizes and recommend that such data simply be presented in the clearest and most direct manner. Accordingly, in this investigation the frequency distributions of cell lengths and widths were determined, but volumes were not calculated. Photomicrographs were placed in a microfilm reader and the lengths and widths of the magnified images of the cells were measured to the nearest 0.5 mm. The final magnification (275×) was such that 1.0 mm. of the image equalled 3.63 μ of the original cell. All of the cells in each photograph were measured, this amounting to about twenty cells in each instance. Five pictures of nondrugged cells just beginning to enter first synchronous division were examined; from the same experiment, five pictures of drugged cells likewise at the onset of first synchronous division were selected.

The data for cell lengths (Fig. 1) show the distribution of cell sizes within the drugged culture to be similar to that within the control. The phenylurethane brought about a shift of the entire range of cell sizes in the direction of smaller cells rather than the formation of a group of abnormally small cells within an otherwise normal cell size distribution. This is evidence that, when ready to begin the first synchronous division, the average drugged cell must have been smaller than the average nondrugged cell. Thus the delay in onset of first synchronous division was not brought about by a requirement for the drugged cells to grow up to the size of the control cells in order to divide.

The data in Table I show that during the temperature cycling the protein content per average nondrugged cell increased about threefold. Thus, protein synthesis and increase in cell volume kept

TABLE I.—THE EFFECT OF PHENYLURETHANE ON CELL VOLUME, PROTEIN, AND DNA CONTENT OF *Tetrahymena pyriformis* GL[a]

Per Average Cell	Log Phase	After Heat Treatment		At Onset of 1st Synchron. Div.	
		Controls	Drugged	Controls[b]	Drugged[c]
Volume ($\mu^3 \times 10^3$)[d]	14.2 ± 0.6	38.9 ± 2.1	29.1 ± 0.9	.....	.....
Ratio to log phase	1.00	2.74	2.05	.....	.....
Per cent of control	.....	.....	76	.....	.....
Volume ($\mu^3 \times 10^3$)[e]	16.8 ± 0.3	.....	.....	45.6 ± 0.2	36.7 ± 2.5
Ratio to log phase	1.00	.....	.....	2.71	2.18
Per cent of control	.....	.....	.....	.....	81
Protein (mcg. × 10^{-3})[f]	1.44 ± 0.06	4.44 ± 0.17	3.41 ± 0.13	.....	.....
Ratio to log phase	1.00	3.09	2.37	.....	.....
Per cent of control	.....	.....	77	.....	.....
Protein (mcg. × 10^{-2})[e]	1.85 ± 0.19	.....	.....	5.37 ± 0.34	4.30 ± 0.11
Ratio to log phase	1.00	.....	.....	2.96	2.38
Per cent of control	.....	.....	.....	.....	81
DNA (mcg. × 10^{-5})[d]	1.69 ± 0.14	3.11 ± 0.18	2.79 ± 0.08	.....	.....
Ratio to log phase	1.00	1.84	1.65	.....	.....
Per cent of control	.....	.....	90	.....	.....
DNA (mcg. × 10^{-5})[e]	.....	.....	.....	2.86 ± 0.49	2.79 ± 0.10
Ratio to log phase	.....	.....	.....	1.69	1.65
Per cent of control	.....	.....	.....	.....	98

[a] 6×10^{-4} *M* phenylurethane added to culture medium before heat treatment. [b] Measured at fifty-nine minutes after the end of the heat treatment. [c] Measured at seventy-eight minutes after the end of the heat treatment. [d] Average of 6 experiments. [e] Average of 2 experiments. [f] Average of 9 experiments.

The standard errors were calculated from the formula:

$$\text{S.E.} = \pm\sqrt{\frac{\Sigma(X - \bar{X})^2}{n(n-1)}}$$

TABLE II.—THE EFFECT OF PHENYLURETHANE ON CELL VOLUME, PROTEIN, AND DNA CONTENT OF *Tetrahymena pyriformis* GL[a]

Per Average Cell	Log Phase	After Heat Treatment	At Onset of 1st Synchron. Div.	
			Controls[b]	Drugged[c]
Volume ($\mu^3 \times 10^3$)[d]	15.9 × 0.2	36.2 ± 1.4	43.5 ± 2.7[e]	40.3 ± 1.8[e]
Ratio to log phase	1.00	2.28	2.74	2.53
Per cent of control	.....	.....	.....	93
Protein (mcg. × 10^{-2})[d]	1.75 ± 0.07	4.39 ± 0.23	4.40 ± 0.26	4.67 ± 0.31
Ratio to log phase	1.00	2.53	2.53	2.68
Per cent of control	.....	.....	.....	106
DNA (mcg. × 10^{-5})[f]	1.94 ± 0.01	.....	4.67 ± 0.12	4.58 ± 0.03
Ratio to log phase	1.00	.....	2.44	2.40
Per cent of control	.....	.....	.....	99

[a] 6×10^{-4} *M* phenylurethane added to culture medium after heat treatment. [b] Measured at approximately sixty minutes after the end of the heat treatment. [c] Measured at approximately sixty-two minutes after the end of the heat treatment. [d] Average of 4 experiments. [e] The average cell volume at onset of synchronous division was statistically different from that after heat treatment according to the *t*-test ($p < .05$). [f] Average of 2 experiments.

The standard errors were calculated from the formula:

$$\text{S.E.} = \pm\sqrt{\frac{\Sigma(X - \bar{X})^2}{n(n-1)}}$$

pace during the heat treatment. Phenylurethane inhibited this protein synthesis to the same extent as it did the volume increase. After cycling was completed, the average protein content of the drugged cell, though increased more than twofold, averaged only 77% of that of the control cell at the same stage (Table I). At the onset of synchronous division, the drugged cells had not yet synthesized as much protein as had the nondrugged (Table I). This correlates with the smaller drugged cell volume observed at the onset of synchronous division.

The deoxyribonucleic acid (DNA) content of control cells was found nearly to double during the heat treatment (Tabie I). Comparable results are in the literature (11, 12). A fourfold increase in DNA content per cell also has been reported (13). When the drugged cultures were temperature-cycled, the average DNA per cell increased by a factor of 1.65. Application of the *t*-test (14) shows that the difference between the DNA content of drugged and control cells was not statistically significant ($p > .05$). At the time of the onset of first synchronous division, there was likewise no significant difference between the DNA content of drugged and control cells (Table I). Apparently, phenylurethane had less effect upon DNA synthesis than upon synthesis of protein and increase in cell size during temperature cycling.

The Effects of 6×10^{-4} *M* Phenylurethane Added After Heat Treatment.—When phenylurethane was added at approximately fifteen minutes after the end of the heat treatment, i. e., to already synchronized cells, the drug showed no significant effect upon cell volume, protein content, or DNA content during the subsequent first lag period. Data in Table II show each of these attributes to

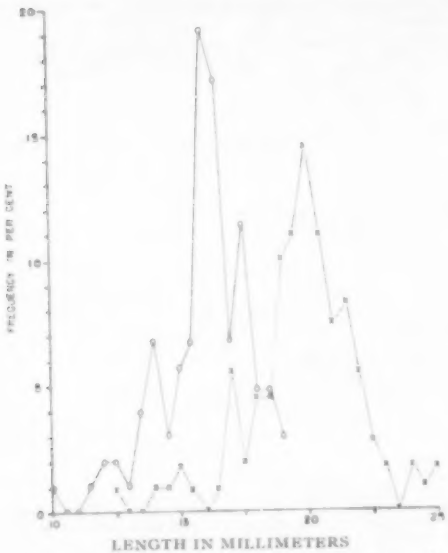

Fig. 1.—The effect of phenylurethane on the distribution of cell lengths; 6×10^{-4} *M* phenylurethane added before heat treatment. X, controls; O, drugged. The lengths presented in the figure are those of the projected cell and can be multiplied by 3.3 to give actual cell lengths in microns.

be equivalent for drugged and control cells at the onset of first synchronous division. It should be pointed out that under these experimental conditions, drugged cells entered into first synchronous division from five to ten minutes later than did the controls, and the magnitude of maximum division index was decreased by about 12% (1). Also, second synchronous division of drugged cells was even more delayed (by about twenty minutes) and its maximum division index was reduced to about 50% of the control value of 0.28 (1).

These data also show that under our experimental conditions there was a 20% increase in cell size, but no synthesis of protein during the first lag period. Christensson (15) has found that protein synthesis stops and that cell protein content may even fall during the first lag period. Sherbaum (11) has reported dry weight to increase by 25% during this interval and that "the mean cell size increases slightly before the first division" (9). In another work (16), he and co-workers found an increase of 8–22% in cell volume to occur between the end of the heat treatment and the onset of synchronous division and that the changes in cell density (dry weight divided by volume) which did occur showed no characteristic pattern. Our data show increase in volume and protein content to parallel during heat treatment and suggest that the cell volume increase during the first lag period could be due to water uptake not associated with the synthesis of protein. Whether growth does or does not take place between the end of the heat treatment and the onset of first synchronous division may be related to the number of exposures to the 34° level. Some workers (9, 15) have used eight temperature shocks as we have; a series of seven shocks (11, 16) has also been used. Furthermore, variations in the degree of nutritional enrichment of the several culture media employed in these investigations may have established different ultimate limitations upon the rate and the total extent of growth possible both during the heat treatment and the first lag period.

In view of the frequently proposed relationship between DNA and cell division, it is interesting that we were unable to show phenylurethane inhibition of DNA synthesis. Drugged cells were found to be delayed in entrance into first synchronous division even though averaging approximately the same DNA content as nondrugged cells (Tables I and II). Our results are perhaps related to those of Ducoff (17) who has reported that X-ray inhibition of the onset of synchronous division occurred when tetrahymena were irradiated after the heat treatment was completed. The fact that the onset of division was delayed even though the tetrahymena had an abnormally large DNA content at the time of irradiation was considered evidence against the hypothesis that X-ray irradiation inhibits cell division by preventing DNA synthesis.

It would appear that cell size *per se* is not a major controlling factor in the initiation of synchronous cell division. The average drugged cell may be smaller than the average nondrugged cell at the time of division (when heat-treated in contact with phenylurethane), or it may be the same size as the nondrugged cell and yet divide later (when phenylurethane is added to already synchronized cells). Prescott has reported that initiation of cell division does not depend on the attainment of a fixed critical size for asynchronously growing tetrahymena (18) or for amebae (19), although ameba cell size may influence the length of the cell cycle (20). Similar evidence indicates that the total cellular protein content does not dictate when synchronous division shall occur. As with DNA, the possible importance of a quantitatively insignificant but very specific protein cannot be dismissed from consideration. It should also be observed that the notable inhibition by phenylurethane of the marked growth and protein synthesis which occurred during the heat treatment was accompanied by pronounced effects upon the subsequent first synchronous division. On the other hand, when the drug was added at the start of the first lag period during which little growth or protein synthesis was found to occur, the effects upon the first synchronous division were quantitatively much lessened.

SUMMARY

1. When tetrahymena were synchronized by heat treatment, the average cell volume and cell protein were increased threefold and the DNA twofold during the period of temperature cycling.

2. When tetrahymena were synchronized by heat treatment in the presence of 6×10^{-4} *M* phenylurethane, the increases in cell volume and

protein occurred to a lesser degree. After temperature cycling, the drugged cells averaged 76% of the volume and 77% of the protein of the nondrugged cells. DNA content of drugged cells was not significantly lower than that of nondrugged. At the onset of their division (eighty minutes after temperature cycling) the drugged cells were smaller, had a lower protein content, and the same DNA content when compared to the nondrugged cells at the onset of their division (sixty minutes after temperature cycling). The phenylurethane did not alter the relative distribution of cell sizes, but did shift the normal range of cell sizes in the direction of smaller cells.

3. When 6×10^{-4} *M* phenylurethane was added to already synchronized tetrahymena shortly after the end of the temperature cycling, the drugged cells were not significantly different from the controls in volume, protein, or DNA at the onset of first synchronous division (about six minutes later for drugged cells than for controls).

4. The implications of the phenylurethane inhibition of cellular synthesis are discussed in relation to its effects upon synchronous divisions.

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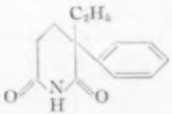
A Quantitative Fluorometric Reaction for Glutethimide*

By R. P. HAYCOCK, P. B. SHETH, and W. J. MADER

A fluorometric pocedure is described for the determination of glutethimide (2-ethyl-2-phenylglutarimide) in tablet formulations which is based on the fluorogen resulting from its reaction with concentrated sulfuric acid containing formaldehyde. The fluorescent intensity is a straight line function of concentration over a wide range. The excitation and fluorescent spectra were determined with a spectrophotofluorometer as an aid in studying the method.

GLUTETHIMIDE[1] is a central nervous system depressant, hypnotic, and sedative. Its structural formula is



An examination of the literature shows a scarcity of methods for its determination.

Using *p*-nitrobenzeneazoresorcinal as the indicator and pyridine or ethylenediamine as the solvent, glutethimide may be titrated as a monobasic acid with sodium methoxide (1). Direct titration of glutethimide in powdered tablet samples is not possible if the tablet excipients are acidic unless it is used in conjunction with other techniques to isolate glutethimide from interfering substances. Goldbaum, Williams, and Koppanyi (2) recognized that glutethimide in strong alkaline solutions possesses a strong absorption maximum at 235 mμ with an absorptivity (1%,1 cm.) of 880, whereas in methanol, glutethimide exhibits a very weak absorption as shown in curve *A*, Fig. 1. The occurrence of strong absorptivity in alkaline solutions is presumably related to resonance of the molecule permitted by ionization of the nitrogen-bound hydrogen atom (3). However, the rapid hydrolysis of gluteth-

* Received April 7, 1960, from the Research Department, Ciba Pharmaceutical Products, Inc., Summit, N. J.
[1] Ciba's trade name for glutethimide is Doriden.

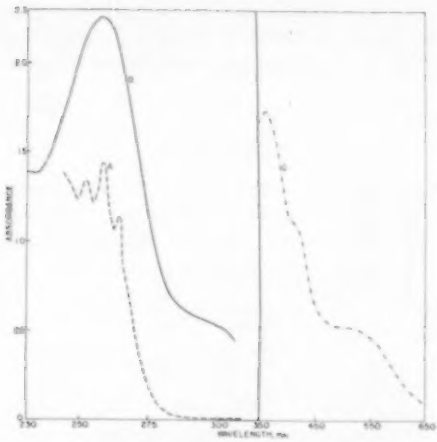


Fig. 1.—Absorption curves of glutethimide. *A*, Glutethimide in methanol 0.4 mg./ml.; *B*, formaldehyde-sulfuric acid fluorogen of glutethimide 0.02 mg./ml.; *C*, formaldehyde-sulfuric acid fluorogen of glutethimide 0.2 mg./ml.

imide in strong alkaline solution is not conducive to its determination in pharmaceutical preparations. Sheppard (4) and associates utilized a method based on the formation of a colored complex between ferric ion and the hydroxamate resulting from the reaction of glutethimide with alkaline hydroxylamine for the determination of the drug in urine. The presence of lactose and anions which complex with ferric ion or salts which form precipitates with ferric ion interfere with the method. Notwithstanding the adaptability of these methods to a particular problem, they present difficulties and ambiguities in the determination of glutethimide in certain tablet formulations.

The authors have observed that glutethimide when heated with concentrated sulfuric acid containing formaldehyde gives a reddish coloration with an intense blue fluorescence. The transformation of glutethimide into a fluorogen with formaldehyde-sulfuric acid reagent is the basis of the method of analysis to be described. Optimum conditions for the development of the fluorescences have been determined, and the applicability of the reaction to related compounds is noted.

EXPERIMENTAL

The fluorescent properties of formaldehyde-sulfuric acid treated glutethimide were studied using the Aminco-Bowman spectrophotofluorometer in order to facilitate the selection of filters for the quantitative measurements. The instrument was calibrated against quinine by the method of Sprince and Rowley (5). The excitation and fluorescent spectra which characterize the fluorogen were obtained using a Moseley model No. 3 flat bed XY recorder as shown in Fig. 2. Measurements were made at room temperature in a quartz cell having a 1-cm. light path using $^1/_{10}$ inch defining slit (band pass = 6 mμ). An RCA 1P21 photomultiplier tube was used to detect the emission. The excitation spectrum is represented by a plot of the exciting wavelength against the intensity of the fluorescent light. The fluorescent spectrum represents the relative intensity of the fluorescent light over the range of 200 to 600 mμ. Excitation maxima were observed at 280 and 365 mμ, and the corresponding maximum fluorescent emission occurred at 450 mμ. The peaks checked within 5–10 mμ on repeated tests.

Absorbance measurements of the fluorogen were made with a Cary recording spectrophotometer as shown in curves *B* and *C*, Fig. 1. A maximum at 257 mμ in the ultraviolet region and a maximum at 365 mμ in the near visible region are indicated. The wavelength of greatest excitation seems to coincide with the strongest absorption band (365 mμ) nearest the fluorescent spectrum band.

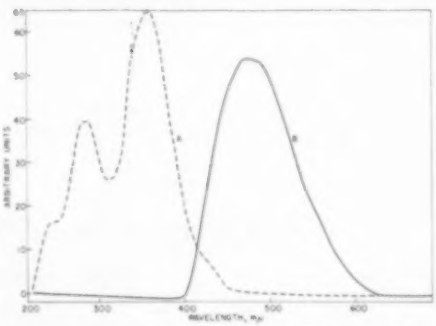


Fig. 2.—Excitation and fluorescent spectra of formaldehyde-sulfuric acid fluorogen of glutethimide. *A*, Excitation scan—fluorescence held at 450 mμ; *B*, fluorescent scan—excitation held at 380 mμ.

PROPOSED QUANTITATIVE PROCEDURE

Reagents.—Unless otherwise indicated, all reagents are reagent grade. Formaldehyde-sulfuric acid reagent: dilute 10 ml. of 37% formaldehyde to 100 ml. with concentrated sulfuric acid with cooling. Prepare fresh daily; diluted sulfuric acid, 50%; dilute 100 ml. of water with 100 ml. of sulfuric acid cautiously with cooling; reference standard glutethimide: use crystalline glutethimide assayed by phase solubility (6), solubility in 50% aqueous methanol is 15.1 mg./Gm.; standard glutethimide solution: prepare standards containing 0.1, 0.3, 0.5, 0.7, and 1.0 mg./ml. in methanol.

Apparatus.—Use a photofluorometer such as the Coleman photofluorometer model 12C with broad band Corning 5970 filter (PC-6) to isolate the exciting radiation (365 mμ) and a combination Corning 3389–4308 filter (PC-1) which transmits 450 mμ to isolate the fluorescent light emitted.

Procedure.—Weigh a counted number of not less than 20 tablets, and reduce them to a fine powder. Transfer an accurately weighed portion of the pow-

der, equivalent to 50 mg. of glutethimide, to a 100-ml. volumetric flask. Dilute to volume with methanol and shake. Centrifuge a portion of the mixture and use the clear supernatant liquid for fluorescent development (concn. = 0.5 mg./ml.).

Pipet 1 ml. of each standard solution of glutethimide, 1 ml. of the sample preparation, and 1 ml. of methanol into separate 50-ml. volumetric flasks. Add 5 ml. of formaldehyde-sulfuric acid reagent, and heat in the oven for twenty minutes at 100° with occasional swirling of the contents of each flask. Cool the flasks to room temperature, dilute to volume with diluted sulfuric acid, adjust the temperature to 25°, and shake well. Allow the flasks to stand for fifteen minutes to permit air bubbles to escape. Adjust the photofluorometer to an arbitrary point, 75, with the highest standard and at zero with the blank using the standard cell supplied with the instrument. Determine the instrument readings on the other standards and the sample preparation. A plot of fluorescent intensity *vs.* concentration, as shown in Fig. 3, is linear. Ascertain the concentration of sample preparation from the graph. In routine analyses it is unnecessary to run several concentrations of standard. Use a standard containing 0.5 mg./ml. and calculate the quantity of glutethimide in mg. in the sample by the formula $(A-B)/(S-B) \times 50$, where A and S are the fluorescent readings of the sample and standard, respectively, and B is the blank.

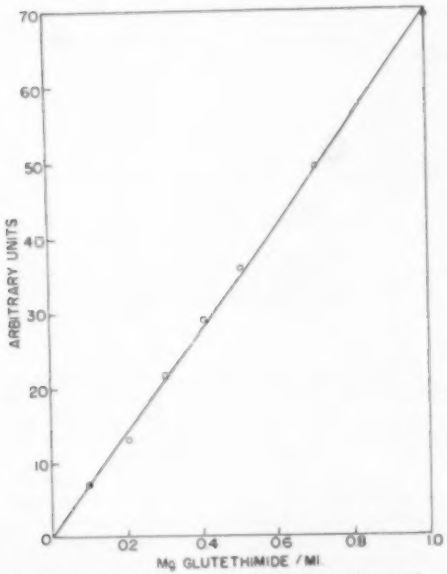


Fig. 3.—Plot of fluorescences of final solution against initial concentration of glutethimide.

DISCUSSION

In order to determine the optimum reaction time for the formation of the fluorogen, the fluorescence of the final solution as a function of heating was investigated. From the curve shown in Fig. 4, it can be seen that at 100° a constant maximum fluorescence is obtained after fifteen minutes. On this basis twenty minutes was selected for the assay procedure. Temperatures below 100° were unsatisfactory, and at room temperature the fluorogen was not formed.

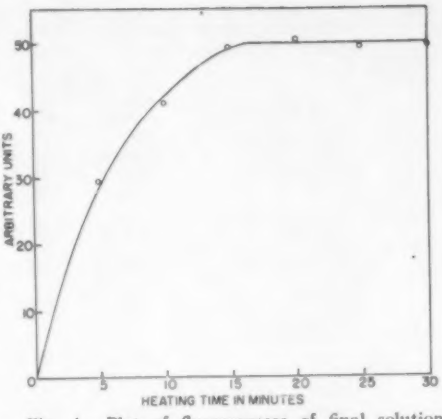


Fig. 4.—Plot of fluorescences of final solution against heating time of reaction. Initial concentration of glutethimide was 0.5 mg./ml.

The behavior of glutethimide toward concentrated sulfuric acid was determined in advance of the addition of formaldehyde, but no fluorescence was detected indicating that the formaldehyde was an essential factor in the reaction. Figure 5 illustrates the effect of the concentration of formaldehyde on the fluorescent intensity. The fluorescence of the solution remained constant and stable for a reagent containing 6 to 20 ml. of formaldehyde for each 100 ml. of sulfuric acid. For convenience, 10 ml. of formaldehyde was used in the reagent for the proposed analytical method. Subsequent experimental

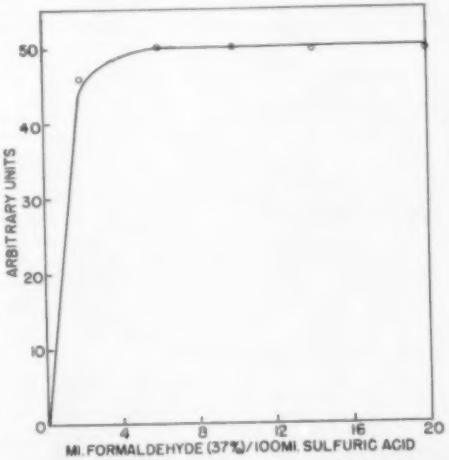


Fig. 5.—Plot of fluorescences of final solution against concentration of formaldehyde. The initial concentration of glutethimide was 0.5 mg./ml.

TABLE I.—COMPARISON OF FLUORESCENT AND COLORIMETRIC PROCEDURES

Sample	Description and Declaration	Glutethimide, % of Declaration: Fluorescent	Glutethimide, % of Declaration: Colorimetric
1	Tablets, 125 mg.	100.6	100.0
2	Tablets, 125 mg.	100.5	100.6
3	Tablets, 250 mg.	100.3	99.6
4	Tablets, 500 mg.	98.5	98.4
5	Tablets, 250 mg. with reserpine, 0.2 mg.	101.5	100.4
6	Tablets, 125 mg. with reserpine, 0.1 mg.	99.5	100.8
7	Tablets, 125 mg. with reserpine, 0.1 mg.	103.3	102.4
8	Tablets, 125 mg. with methylphenidate,[a] 5 mg.	101.5	99.9
9	Tablets, 125 mg. with methylphenidate, 10 mg.	100.7	100.0
10	Capsules, 125 mg.	104.0	107.0

[a] Ciba's trade name for methylphenidate is Ritalin.

data indicated that trioxymethylene could be substituted for the formaldehyde solution.

The addition of water to the fluorogen formed on heating glutethimide with formaldehyde-sulfuric acid reagent destroys the fluorescence. However, the final solution may be diluted to volume with diluted sulfuric acid in concentrations greater than 40% without adversely affecting the intensity of the fluorescence. In the assay procedure 50% sulfuric acid was more suitable and was used in the proposed method.

The authors have analyzed several lots of tablets containing glutethimide and combinations with other drugs by both the hydroxamic acid colorimetric procedure and the proposed fluorometric technique. The colorimetric procedure required preliminary extraction with chloroform to separate glutethimide from lactose. The summary of these analyses, as shown in Table I, indicates that the two methods yield concordant assay values. Common tablet excipients such as starch, powdered sugar, lactose, stearic acid, magnesium stearate, talcum, and magnesium aluminum stearate do not interfere with the

TABLE II.—FLUORESCENCE OF GLUTETHIMIDE AND SOME RELATED COMPOUNDS

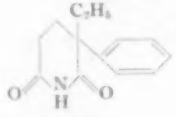
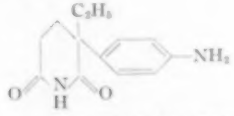
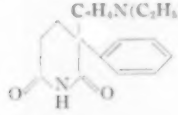
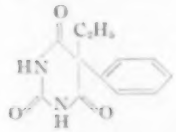
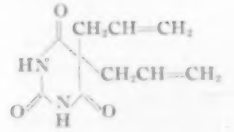
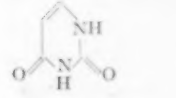
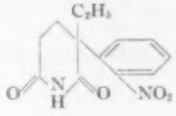
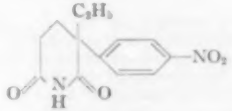
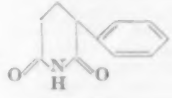
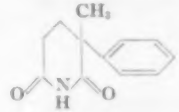
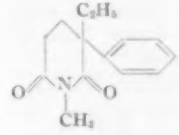
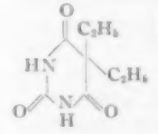
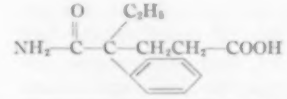
Compound	Structure	Relative Fluorescent Intensity
Glutethimide		100
Aminoglutethimide[a]		Nil
Phenglutarimide	$C_6H_5N(C_2H_5)_2 \cdot HCl$ 	23
Phenobarbital		73
Diallylbarbituric acid[a]		23
Uracil		Nil
Benzene		Nil (colored)

TABLE II. (*continued*)

Compound	Structure	Relative Fluorescent Intensity
2-Ethyl-2-(*o*-nitrophenyl)-glutarimide		Nil
2-Ethyl-2-(*p*-nitrophenyl)-glutarimide		Nil
2-Phenylglutarimide		78
2-Methyl-2-phenylglutarimide		96
2-Ethyl-N-methyl-2-phenylglutarimide		100
Diethylbarbituric acid		Nil
4-Ethyl-4-phenylglutaramic acid		10

[a] Ciba's trade name for aminoglutethimide is Elipten. [b] Ciba's trade name for phenylglutarimide is Aturban. [c] Ciba's trade name for dialylbarbituric acid is Dial.

fluorescent technique. The totality of results on several commercial lots performed in triplicate showed a precision of ±2%.

In order to determine the selectivity of the reaction and the effect of various moieties on the molecule, a number of related compounds were studied, as summarized in Table II. The proposed assay is applicable to certain structurally related derivatives containing an unsubstituted phenyl group. Except for diallylbarbituric acid, the reaction does not occur with compounds lacking a phenyl group or compounds containing a substituted phenyl moiety. The order of magnitude of fluorescence depends upon certain groups in the basic molecule which may retard or prevent the fluorogenic reaction. The procedure is applicable for the determination of glutethimide in the presence of its degradation product, 4-ethyl-4-phenylglutaramic acid, since the latter yields only one-tenth the fluorescence of glutethimide. Although the chemistry of the reaction of glutethimide with formaldehyde-sulfuric acid reaction is obscure, it seems likely that concentrated sulfuric acid brings about a condensation of the compound with formaldehyde and then oxidizes the resulting compound to a fluorescent compound. Phosphoric acid which behaves like sulfuric acid with respect to dehydration but, in contrast, never acts as an oxidant does not form the fluorescent compound in the presence of formaldehyde.

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Adaptation of the Chromotropic Acid Method to the Assay of Spans*

By LACEY GATEWOOD, Jr., and HORACE D. GRAHAM

The chromotropic acid method for the determination of formaldehyde has been adapted to the direct and quantitative determination of the Span surfactants. The proposed method involves saponification of the particular Span to yield sorbitol and the particular fatty acid. The sorbitol portion is then oxidized by periodate to yield formaldehyde which is coupled with chromotropic acid to yield a wine color with an absorption maximum at 570 mμ. This procedure has been found to be rapid and simple with a high degree of sensitivity.

THE SPANS are nonionic surfactants produced through the esterification of various fatty acids and sorbitan, the dehydrated form of sorbitol, a hexahydroxy alcohol. They are widely used in pharmaceutical products and their reactions influence the efficacy and stability of pharmaceutical formulations. Their many applications, including some recent ones (3), allegedly involve an interaction between the surfactant and specific ingredients. The concentration of these surfactants used, in most cases, is very low. The exact nature of the mechanisms whereby they exert their influence can be ascertained, therefore, only if rapid, precise, and sensitive methods for their determination are readily available. To date, no rapid colorimetric method for assay of this class of surfactants has been reported (6). Therefore, these studies were initiated to develop a rapid colorimetric method for their quantitative determination exploiting the chromotropic acid method for formaldehyde (4) which has been modified and adapted to, among other things, the estimation of mannitol and sorbitol (2, 8).

If subjected to alkali saponification, the Spans, in aqueous solution, should yield sorbitol and the particular fatty acid. The sorbitol portion should, therefore, be estimable by the chromotropic acid method to give a direct measure of the concentration of the particular Span. This premise was experimentally verified and constitutes the basis of this paper.

* Received November 27, 1959, from the George Washington Carver Foundation, Tuskegee Institute, Alabama.

The authors wish to acknowledge gifts of the various samples of Spans used from the Atlas Powder Co., Wilmington, Del.

EXPERIMENTAL

Materials and Reagents.—Span 20, sorbitan monolaurate, lot 333; Span 40, sorbitan monopalmitate, lot 8068c; Span 60, sorbitan monostearate, lot 7615c; all obtained from the Atlas Powder Co., Wilmington, Del. Potassium hydroxide, 0.5 *M*, U. S. P., Fisher Scientific Co. Periodic acid reagent, 0.0075 *M*. Stannous chloride reagent, 0.0035 *M*. Chromotropic acid reagent, disodium salt, practical grade, Eastman Kodak Co., Rochester 3, N. Y. These reagents were prepared according to West and Rapoport (8).

Equipment.—Coleman universal spectrophotometer, model 14; 20-ml. Pyrex-stoppered test tubes.

GENERAL PROCEDURE

Saponification Step.—In all preliminary studies, Span 20 only was used. One gram was weighed into a 250-ml. Erlenmeyer flask. To this 50 ml. of 0.5 *M* potassium hydroxide were added. The flask was placed in a boiling water bath for twenty minutes and after cooling the contents were quantitatively transferred to a 1,000-ml. volumetric flask and made up to volume with distilled water. When necessary, further dilutions were made with distilled water to obtain solutions for the subsequent oxidation and color development steps.

Oxidation of the Sorbitol Produced Through Saponification and Color Development With Chromotropic Acid.—Solutions containing the equivalents of 300, 400, and 500 mcg./ml. of the original Span were made up from the saponified sample by appropriate dilution with distilled water. One milliliter of each of these solutions was pipetted into duplicate 20-ml. glass-stoppered Pyrex test tubes. A control (in duplicate) containing distilled water instead of the saponified Span was included. Periodic acid reagent, 0.5 ml., was added to each tube and the contents were thoroughly mixed. The tubes were allowed to stand for eight minutes at room temperature (28 ± 1°). At the end of this period, 0.5 ml. of the stannous chloride reagent was added to each tube, the contents again mixed well, and the tubes placed in a cold water bath. Six milliliters of the chromotropic acid reagent and 2 ml. of distilled water were added to each tube and the tubes immersed in a boiling water bath for thirty minutes. After removal and cooling, the color intensity was measured at 570 mμ. Confirmation of the wavelength of maximum absorption was obtained by measuring the absorbance over the wavelength range of 400–700 mμ and, as shown in Fig. 1, is at 570 mμ, which is the same as that for formaldehyde, as found by Bricker and Johnson (1), and for sorbitol, as previously established (8) and reproduced here. Span 40 and Span 60 gave absorption spectra similar to that of Span 20.

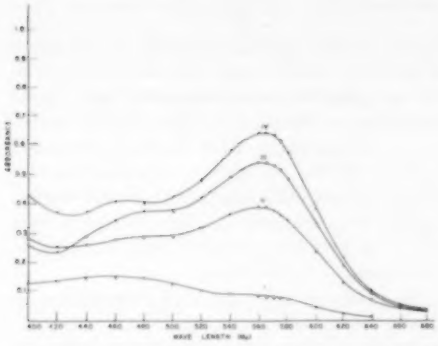

Fig. 1.—Absorption spectra of chromotropic acid complexes of formaldehyde and the periodiate oxidation products of sorbitol and Span 20. I, blank; II, Span 20; III, sorbitol; IV formaldehyde.

The concentration of a particular Span in an unknown can be determined in exactly the same way and calculated from a standard curve for the Span. If the amount of formaldehyde produced is used as the basis of calculation, then this can be determined from a standard curve for the formaldehyde-chromotropic acid reaction, established according to the general procedure but omitting the saponification step.

Influence of Saponification Time.—Duplicate solutions containing 400 mcg. of Span 20 were saponified for times indicated in Table I and the intensity of the color developed measured at 570 mμ.

The results indicate that the difference in per cent transmission for saponification times of ten to thirty minutes fell well within the range of experimental error. At and above forty minutes, color development was more intense but no constancy was observed. In view of the constant response over the lapse of ten to thirty minutes, a saponification period of thirty minutes was chosen.

TABLE I.—EFFECT OF SAPONIFICATION TIME ON COLOR INTENSITY

Saponification Time, min.	Transmission, %	Selected Time, min.
10	27.8	30
20	27.5	
30	27.0	
40	25.2	
50	23.2	

RELATIONSHIP BETWEEN COLOR INTENSITY AND CONCENTRATION OF SPAN

After the optimal saponification time was established, aliquots of the saponified Span corresponding to 0, 100, 200, 300, 400, and 500 mcg. of the original Span were placed in duplicate ground-glass-stoppered test tubes. The samples were oxidized for eight minutes, the color developed according to the general procedure, and its intensity measured at 570 mμ. Preliminary experiments had shown that for color development an oxidation time of eight minutes, 0.5 ml. of the periodate solution containing 3.75×10^{-6} moles of potassium periodate, 1.75×10^{-5} moles of stannous chloride, 11.25×10^{-6} moles of chromotropic acid, and a heating time of thirty minutes were optimal. These limits for this system correspond well with those established by Bricker and Johnson (1) for formaldehyde.

When the logarithm of the per cent transmission as recorded in Table II is plotted as a function of the concentration of added Span, a linear relationship results. This indicates that a quantitative relationship exists and that Beer's law is being obeyed.

TABLE II.—RELATIONSHIP BETWEEN CONCENTRATION OF SPAN 20 AND THE INTENSITY OF THE COLOR DEVELOPED

Concentration of Span 20, mcg./10 ml.	Transmission, %		
	Expt. I	Expt. II	Average
0	87.0	85	86.0
100	63.9	62	63.0
200	46.9	44.8	45.8
300	33.4	32.8	33.1
400	24.5	24.7	24.6
500	19.0	18.0	18.5

Recovery of Span 20 from Mixtures Containing Anionic Surfactants.—Since pharmaceuticals may contain anionic surfactants, it was thought of interest to study recovery from a mixture containing some common forms. For this, 1.0 Gm. of Span 20, 0.5 Gm. of sodium lauryl sulfate, 1.0 ml. of sodium tetradecyl sulfate, and 0.5 Gm. of sodium lauryl sulfonate were mixed with 50 ml. of 0.5 *M* potassium hydroxide in a 125-ml. Erlenmeyer flask. The mixture was placed in a boiling water bath for thirty minutes. Aliquots of the mixture calculated to contain 10, 20, and 40 mcg. of the added Span 20 were oxidized and the color developed as in previous experiments.

The results summarized in Table III show that high recovery of Span 20 was achieved in the presence of the anionic detergents used.

TABLE III.—RECOVERY OF SPAN 20 FROM MIXTURES OF ANIONIC SURFACTANTS

Amount Added, mcg.	Span 20 Recovered, mcg.	Recovered, %
10	10	100.0
20	18.9	94.5
40	38.8	97.0

DISCUSSION

Experimental evidence has been obtained to demonstrate that the chromotropic acid method for the determination of formaldehyde can be adapted to the direct assay of the "Span" class of surfactants. Preliminary alkali saponification of the Span is necessary in order to release the sorbitan moiety for subsequent oxidation to formaldehyde. For optimal conditions in the system employed, a saponification time of thirty minutes, an oxidation time of eight minutes, a level of 3.75×10^{-6} moles of potassium periodate, 1.75×10^{-5} moles of stannous chloride,

11.25×10^{-6} moles of chromotropic acid, and a heating time of thirty minutes were selected.

The similarities in the absorption spectra and visual appearance for identically developed colors using formaldehyde, sorbitol, and the Spans confirm that formaldehyde is actually produced after saponification and oxidation. Furthermore, evidence was obtained that the formaldehyde produced from the saponification product of the Span can be determined directly by the chromotropic acid method. Although only three Spans were used, since the other commercially available ones, namely, Span 65 (sorbitan tristearate), Span 80 (sorbitan monooleate), and Span 85 (sorbitan trioleate), all contain the sorbitan moiety, the test can be considered as a class test for the Spans.

Since many formulations are combinations of anionics and nonionics, recovery tests were attempted from mixtures. In the presence of anionics, good recovery was achieved (Table II). Weeks, Ginn, and Baker (7) analyzed for nonionics gravimetrically after ion exchange separation to remove the anionic type. The small quantities of nonionics in most preparations caused them some concern in their otherwise successful approach. The proposed direct spectrophotometric method could supplement the ion-exchange approach. Attempts to make direct assays in the presence of cationic surfactants were unsatisfactory. However, by use of ion exchange resin such cationic surfactants could be removed prior to analysis by the proposed method. Rosen (5) has also outlined a simple, useful procedure for the separation of nonionic surfactants from mixtures with anionics by ion exchange. Polyoxyethylene types of nonionic surfactants will also give a positive test with chromotropic acid. However, as noted by Rosen (5), the oxonium salt of polyoxyethylene compounds can be precipitated by large anions such as cobaltothiocyanate, or phosphomolybdate ion. This, then, could offer a possible separation of the Tweens from the Spans after removal of anionic and cationic surfactants by ion exchange resins.

SUMMARY

The chromotropic acid method for the determination of formaldehyde has been adapted to the quantitative determination of the "Span" surfactants. The particular Span is first saponified to release the sorbitol moiety. Controlled oxidation of the sorbitol by periodate leads to the production of formaldehyde which is then coupled with chromotropic acid to give a wine color, the intensity of which is proportional to the amount of Span present.

Optimum conditions for determination are as follows: a saponification time of thirty minutes, an oxidation time of eight minutes, a level of 3.75×10^{-4} moles of chromotropic acid, and a heating time of thirty minutes for developing the color. These conditions are specified for a total volume of 10 ml. in the system.

Satisfactory recovery was achieved in the presence of anionic surfactants, but recovery in the presence of cationic surfactants was unsatisfactory. For reproducible results it is recommended that all reagents be prepared fresh each day.

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Notes

Note on X-ray Crystallographic Data on Some Alkaloids from *Vinca rosea* Linn.*

By ANN VAN CAMP

A NUMBER of compounds have been obtained from the pantropical plant, *Vinca rosea* Linn. An account of the preparation and characterization of the alkaloids from this plant has been given by Svoboda, Neuss, and Gorman (1).

Crystallographic data on two alkaloids from this plant, reserpine and ajmalicine, have been published by Rose (2, 3). Previously unpublished X-ray crystallographic data on nine alkaloids from this plant are presented here.

EXPERIMENTAL

Crystals of catheranthine, catheranthinol, and lochnericine suitable for crystallographic work were crystallized from methanol; leurosine was crystallized from acetonitrile; vincaleucoblastine etherate, vindoline, and vindolicine were crystallized from ether; vincaleucoblastine methanolate was crystallized from methanol; perivine hydrochloride was crystallized from methanol-ether mixture; and virosine was crystallized from chloroform-methanol mixture.

The X-ray powder diffraction data on all samples except perivine hydrochloride were obtained with chromium radiation and vanadium filter, using a wavelength value of 2.2896 Å. in the calculations. Copper radiation and nickel filter with a wavelength value of 1.5405 Å. were used to obtain the data on perivine hydrochloride. A standard Norelco powder camera, 114.6 mm. in diameter, was used.

The single crystal data were obtained using nickel filtered copper radiation and a Weissenberg camera.

DISCUSSION

Table I gives X-ray single crystal data. Perivine hydrochloride and vindoline give optical biaxial figures. From this fact and from observation of the *hk0* intensities, these two alkaloids cannot have higher than orthorhombic symmetry in spite of the apparent equality of the *a* and *b* cell dimensions.

Table II gives X-ray powder diffraction data. Included in this table are data for three alkaloids, catheranthine, virosine, and vincaleucoblastine methanolate, for which no suitable single crystals were available.

TABLE I.—X-RAY SINGLE CRYSTAL DATA

Compound	System	Cell Dimensions	Axial Ratios *a:b:c*	Density	Formula Wt. Per Cell
Catheranthinol $C_{20}H_{24}N_2O$	Ortho.	$a = 12.00$ $b = 13.52$ $c = 10.48$	0.8876:1:0.7751	1.210 Flotation 1.207 X-ray	4
Leurosine $C_{46}H_{58}N_4O_9 \cdot 8H_2O$	Ortho.	$a = 20.03$ $b = 26.63$ $c = 9.44$	0.7522:1:0.3545	1.262 Flotation 1.261 X-ray	4
Lochnericine $C_{21}H_{24}N_2O_3$	Ortho.	$a = 13.40$ $b = 13.73$ $c = 9.96$	0.9760:1:0.7254	1.287 Flotation 1.280 X-ray	4
Perivine·HCl $C_{20}H_{24}N_2O_3 \cdot HCl$	Ortho.	$a = 15.85$ $b = 15.85$ $c = 7.59$	1:1:0.4789	1.303 Flotation 1.307 X-ray	4
Vincaleucoblastine etherate $C_{46}H_{58}N_4O_9 \cdot (C_2H_5)_2O$	Mono.	$a = 22.57$ $b = 21.95$ $c = 19.60$ $\beta = 95°36'$	1.0283:1:0.8930	1.224 Flotation 1.215 X-ray	8
Vindolicine $C_{25}H_{32}N_2O_6$	Mono.	$a = 15.94$ $b = 10.61$ $c = 14.03$ $\beta = 95°$	1.5024:1:1.1327	1.288 Flotation 1.284 X-ray	4
Vindoline $C_{25}H_{32}N_2O_6$	Ortho.	$a = 15.61$ $b = 15.68$ $c = 9.55$	0.9955:1:0.6090	1.279 Flotation 1.299 X-ray	4

* Received March 7, 1960, from the Lilly Research Laboratories, Eli Lilly and Co., Indianapolis 6, Ind.

The author is grateful to Dr. G. H. Svoboda, Dr. N. Neuss, and Dr. M. Gorman for supplying the compounds from which these data were obtained and to Dr. R. R. Pfeiffer for technical advice.

TABLE II.—X-RAY POWDER DIFFRACTION DATA

Catheranthine
$C_{21}H_{24}N_2O_3 \cdot H_2O$

d	I/I_1	d	I/I_1
10.51	1.00	4.94	0.40
9.43	0.80	4.85	0.40
8.74	1.00	4.71	0.40
7.12	0.20	4.40	0.40
5.65	0.40	4.32	0.40
5.49	0.40	3.49	0.20
5.25	0.40		

Catheranthinol
$C_{21}H_{24}N_2O$

d	I/I_1	hkl	d	I/I_1	hkl
9.16	0.30	110	3.75	0.30	212
8.17	0.40	011	3.65	0.08	230
6.78	1.00	020	3.50	0.04	320
5.24	0.80	002	3.40	0.02	040
5.08	0.20	121	3.29	0.04	321
4.90	0.12	012	3.17	0.02	
4.69	0.20	102	3.08	0.08	
4.16	0.30	031	2.96	0.02	
3.90	0.04	131	2.89	0.04	

Leurosine
$C_{46}H_{58}N_4O_9 \cdot 8H_2O$

d	I/I_1	hkl	d	I/I_1	hkl
15.8	0.50	110	5.25	0.30	141
13.4	0.40	020	4.94	0.05	410
11.0	0.50	120	4.73	0.10	250
9.81	0.20	200	4.61	0.30	102
9.29	0.20	210	4.13	0.05	032
8.77	0.05	101	4.03	0.05	440
8.10	1.00	220	3.93	0.10	
7.18	0.50	121	3.76	0.10	
6.59	0.75	211	3.66	0.05	
6.34	0.20	140	3.51	0.05	
6.09	0.20	221	3.38	0.05	
5.47	0.10	041	3.33	0.05	
			3.17	0.05	

Lochnericine
$C_{21}H_{24}N_2O_3$

d	I/I_1	hkl	d	I/I_1	hkl
9.66	0.08	110	3.42	0.08	040
7.95	0.08	101	3.23	0.08	410
6.88	1.00	020	3.16	0.01	141
6.08	0.15	120	3.03	0.02	240
5.62	0.15	021	2.99	0.02	420
5.16	0.40	121	2.87	0.02	421
4.83	0.15	220	2.73	0.01	050
4.59	0.40	030	2.63	0.01	510
4.32[a]	0.40	221, 130	2.55	0.01	511
3.97	0.15	131	2.47	0.01	251
3.78	0.15	040, 320	2.36	0.01	152
3.54	0.15	231			

Perivine hydrochloride
$C_{21}H_{24}N_2O_3 \cdot HCl$

d	I/I_1	hkl	d	I/I_1	$h\,k\,l$
11.32	0.20	110	3.89	0.20	140, 410
8.03	1.00	020, 200	3.76	0.10	330
6.96	1.00	101, 011	3.64	0.10	112
6.34	0.10	111	3.49	0.10	141, 411
5.62	0.30	220	3.38	0.75	331
5.09	0.10	130, 310	3.06	0.30	132, 312
4.56	0.20	221	2.98	0.05	250, 520
4.42	0.20	230, 320	2.90	0.10	232, 322
4.22	0.30	131, 311	2.77	0.05	251, 521

Vincaleucoblastine etherate
$C_{46}H_{58}N_4O_9 \cdot (C_2H_5)_2O$

d	I/I_1	hkl	d	I/I_1	hkl
11.32	0.03	200	4.48	0.07	$40\bar{3}$, 430
9.77	1.00	002	4.35	0.07	204
9.16[a]	0.13	201, $21\bar{1}$	4.17	0.13	$43\bar{2}$, 151
8.65	0.67	102, 121	4.01	0.27	$25\bar{1}$
7.48	0.50	$22\bar{1}$	3.87	0.03	$50\bar{3}$
7.16	0.33	310	3.68	0.07	
5.97	0.13	$31\bar{2}$, 222, $23\bar{1}$	3.56	0.13	
5.84	0.13	$20\bar{3}$	3.43	0.13	
5.50	0.27	040	3.22	0.03	
5.26	0.07	330	3.01	0.07	
5.08	0.07	$40\bar{2}$	2.89	0.07	
4.82	0.27	$24\bar{1}$			
4.59	0.20	$20\bar{4}$			

Vincaleucoblastine methanolate
$C_{46}H_{58}N_4O_9 \cdot 2CH_3OH \cdot H_2O$

d	I/I_1	d	I/I_1
18.7	0.13	4.58	0.20
14.2	0.13	4.38	0.20
10.77	0.13	4.21	0.13
9.81	0.27	4.00	0.20
9.33	0.67	3.76	0.03
8.59	0.67	3.57	0.13
7.79	0.07	3.44	0.13
7.44	0.07	3.34	0.03
7.16	1.00	3.19	0.03
5.84	0.33	2.99	0.03
5.64	0.13	2.79	0.03
5.43	0.27	2.62	0.03
4.79	0.27		

Vindolicine
$C_{25}H_{32}N_2O_5$

d	I/I_1	hkl	d	I/I_1	hkl
14.04	0.60	001	3.50	0.20	004
9.92	0.40	101	3.29	0.16	
8.02	1.00	200	3.21	0.02	230
7.14	0.20	201	3.15	0.02	
6.99	1.00	002	3.08	0.04	
6.78	0.20	201	3.02	0.04	
6.31	0.40	210	3.00	0.04	
5.41	0.80	$20\bar{2}$	2.87	0.02	
5.30	0.08	020	2.70	0.02	
5.14	0.08	301	2.64	0.12	
4.94	0.30	021	2.57	0.04	
4.40	0.02	$30\bar{2}$, 220	2.55	0.04	
4.25	0.60	013	2.47	0.08	
4.00	0.16	400	2.45	0.02	
3.92	0.16	$40\bar{1}$	2.33	0.08	
3.80	0.40	312	2.11	0.04	
3.75	0.04	410	2.01	0.04	
3.69	0.20	321			

TABLE II. (*continued*)

Vindoline $C_{25}H_{32}N_2O_6$			Virosine $C_{23}H_{30}N_2O_4$			
d	I/I_1	*hkl*	*d*	I/I_1	*d*	I/I_1
11.2	0.33	110	16.83	0.01	3.13	0.01
8.20	1.00	101, 011	8.91	1.00	2.99	0.04
7.86	1.00	200, 020	8.22	0.40	2.91	0.04
7.25	0.50	111	7.79	0.30	2.88	0.04
6.09	0.20	021, 201	6.04	0.01	2.79	0.08
5.66	0.27	121	5.71	0.75	2.66	0.04
5.01	0.27	130, 310	4.89	0.75	2.61	0.04
4.80	0.07	221, 002	4.39	0.10	2.49	0.06
4.58	0.27	301, 102, 012	4.24	0.15	2.35	0.01
4.40	0.50	131, 311, 112	4.07	0.20	2.31	0.02
3.95	0.50	231, 321, 122	3.92	0.04	2.28	0.02
3.81	0.27	140, 410	3.87	0.06	2.22	0.01
3.63	0.20	222	3.72	0.04	2.19	0.01
3.53	0.07	240, 420, 141, 411, 302	3.40	0.15	2.12	0.01
3.45	0.13	331, 132, 213	3.19	0.02		
3.38	0.03					
3.20	0.03					
3.07	0.13					
2.92	0.03					
2.80	0.03					
2.76	0.03					
2.68	0.03					
2.62	0.03					

a Indicates broad line.

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Note on the Occurrence of Taxine in *Taxus brevifolia**

By V. E. TYLER, Jr.

A small amount of an alkaloid isolated from the fresh needles of Pacific yew was shown to be amorphous taxine. No cyanogenetic glycoside could be detected in this plant material. The chemotaxonomic significance of these findings is discussed.

A RECENT SURVEY of the chemotaxonomic significance of the occurrence of alkaloids and cyanogenetic glycosides in the genera *Taxus*, *Cephalotaxus*, and *Torreya* has been conducted by Hegnauer (1). All of the species and varieties of *Taxus* investigated were found to contain alkaloids which were shown chromatographically to be identical with taxine, but these alkaloids were distinctly different from those present in *Cephalotaxus*. Many species and varieties of *Taxus* proved to be cyanogenetic, but all species of *Cephalotaxus* and *Torreyea* investigated, as well as some varieties of *Taxus*, were noncyanogenetic.

Taxus brevifolia Nutt., the Pacific or western yew, was not one of the species investigated by Hegnauer, although he concluded on the basis of his survey that the complex alkaloidal mixture, taxine, probably occurs in all species of *Taxus*. This statement appeared to be of special interest in view of the report of Jones and Lynn (2) that *T. brevifolia* contains no alkaloid and probably no glycoside. Consequently, it was deemed worth while to re-examine this plant in an attempt to verify the chemotaxonomic significance of taxine in the genus *Taxus*.

EXPERIMENTAL

Isolation of an Alkaloid.—Fresh needles and small twigs of *Taxus brevifolia* collected in October near the summit of Snoqualmie Pass, Washington, were coarsely comminuted by passage through an Abbe mill No. 000. A 1,150-Gm. quantity of this plant material was placed in a large beaker and allowed to macerate overnight with 2 L. of 2% hydrochloric acid. It was then divided into portions which were homogenized in a Waring Blendor together with appropriate volumes of an additional 2-L. quantity of 2% hydrochloric acid. The acid extract was separated from the resulting mash by expression through muslin in a hand press. The 3 L. of extract thus obtained was concentrated under reduced pressure in a flash evaporator at 55° to a volume of 800 ml. which was rendered alkaline by the addition of ammonium hydroxide. After extraction in a separatory funnel with three successive 200-ml. portions

* Received January 22, 1960, from the College of Pharmacy, University of Washington, Seattle 5.

of chloroform, the chloroform extracts were combined and evaporated to dryness on a steam bath. This residue was redissolved in 30 ml. of ether which was then shaken out with three 5-ml. portions of hydrochloric acid.

In order to effect a further purification of the alkaloid, the combined hydrochloric acid extracts were rendered alkaline with ammonium hydroxide, re-extracted with three 5-ml. portions of ether, these extracts combined and re-extracted three times with a total of 10 ml. of 2% hydrochloric acid. The alkaloid was precipitated from this solution by the addition of ammonium hydroxide, removed from the chilled solution by filtration, and dried in a desiccator over anhydrous calcium chloride. Yield of the alkaloid was 8.9 mg. or 0.00077% of the starting material, calculated on a fresh weight basis.

Identification of Amorphous Taxine.—Since taxine is such a complex mixture of alkaloids which are subject to decomposition, Graf and Bertholdt (3) have concluded that the melting point of the compound has absolutely no relationship to its purity. Likewise, the optical activity displayed by a solution of taxine varies according to the time of harvest of the plant and the botanical source of the compound. Consequently, these classical analytical properties could not be employed in the evaluation of the alkaloid isolated from *T. brevifolia*.

The benzidine and Prussian blue tests for nitrogen (4) yielded negative results when applied to a solution prepared from the products of sodium fusion of small quantities (<3 mg.) of the compound, but the same tests were also negative when applied to similar quantities of known taxine.[1] Subsequently, the isolated product was subjected to a quantitative microanalytical procedure for nitrogen. The percentages of carbon and hydrogen were also determined, although this was considered as a secondary objective, and the quantity of sample available for this determination was very small (1.811 mg.). This may explain the slight discrepancy in the percentage of carbon found in the sample.

Anal.[2]—Calcd. for $C_{31}H_{41}NO_{10}$: C, 66.34; H, 7.67; N, 2.09. Found: C, 61.26; H, 7.72; N, 2.15.

The isolated alkaloid also gave positive reactions to a number of colorimetric tests for taxine. A deep red color was formed when it was treated with concentrated sulfuric acid, and when the Salkowski test was applied, a reddish-orange color was formed in the acid layer and a pink color in the chloroform layer (5). When the alkaloid was spotted on filter paper, sprayed with hydrochloric acid, and heated for a few minutes at 100°, it exhibited the characteristic bluish-red color and yellow fluorescence described by Graf (6). When a similar spot on filter paper was sprayed with diluted Wagner's reagent, it gave a citron-yellow color typical of taxine.

Hegnauer (1) has shown that when amorphous taxine is subjected to paper chromatography in a number of common solvent systems it does not separate into its components but is detectable as a single, rather broad spot. Quantities of the isolated alkaloid were spotted on 3 inch × 12 inch sheets of Whatman No. 1 filter paper, not only as the single compound but also in combination with authentic taxine and with a purified alkaloidal extract freshly prepared from *Taxus baccata* L. These sheets were formed ascendingly for a period of five to six hours with the following solvent systems: *n*-butanol-hydrochloric acid-water (100-10-saturation), *n*-butanol-acetic acid-water (4-1-5), and *n*-propanol-1 *N* ammonia (4-1).

The sheets were sprayed with Munier's reagent (7) which gave reddish-orange spots with taxine and with the isolated alkaloid. The R_f values of the three test substances were identical in each of the three solvent systems employed:

System	Average R_f
Butanol-acetic acid-water	0.79
Butanol-hydrochloric acid-water	0.82
Propanol-ammonia	0.90

Test for Cyanogenetic Glycoside.—The Grignard-Mirande qualitative test for hydrogen cyanide was carried out as described by Hegnauer (8) but employing a double quantity (10 Gm.) of finely chopped, fresh needles of *T. brevifolia*. After incubating for forty-eight hours at 37°, the sodium picrate paper gave no indication of a positive reaction.

CONCLUSIONS AND DISCUSSION

On the basis of the results of the elemental analyses and various color reactions as well as R_f values obtained in three different wash liquids, it was concluded that the alkaloid isolated from *Taxus brevifolia* was amorphous taxine. The existence of this alkaloid in this species of *Taxus* in small (0.00077% of fresh weight) but definite amounts is thus confirmed. This is in contradiction to the earlier report of Jones and Lynn (2) which indicated the absence of alkaloids in *T. brevifolia*.

This discrepancy may be explained, at least in part, by the relatively small amount of alkaloid present in this species, and by the fact that Jones and Lynn based their conclusion on the negative character of sodium-fusion tests for nitrogen. In this study it has been shown that such tests do yield negative results when applied to small amounts of amorphous taxine.

The presence of this alkaloid is of considerable interest from the chemotaxonomic viewpoint, since it confirms the reliability of taxine as a genus characteristic and substantiates Hegnauer's postulation to this effect by correcting a misconception present in the older literature. Furthermore, it supports the observation (1) that the smallest quantities of alkaloid are found in those species and varieties of *Taxus* which lack cyanogenetic glycosides, since glycosides of this type were found to be absent in this alkaloid-poor species.

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[1] A generous sample of amorphous taxine was supplied through the courtesy of Dr. E. Graf, Würzburg, Germany.

[2] Analyses carried out by Drs. G. Weiler and F. B. Strauss, Microanalytical Laboratory, Oxford, England.

Note on the Disappearance of Pharmacologic Activity*

By JOSEPH V. SWINTOSKY and F. M. STURTEVANT

THE CONCEPT of exponential excretion of drugs is not new (1–4); however, applications of this concept to pharmaceutical practice are of relatively recent vintage (5–8). Although most workers have studied only blood and urine levels, Hill (4) was one of the first to recognize the exponential character of the disappearance of some pharmacologic responses. In the assessment of various pharmaceutical forms of a drug, it is preferable to use performance indexes derived from pharmacologic responses, especially when they have some correlation to clinical therapeutic responses. In addition, such responses reflect the action of the original drug as well as all active metabolites. The present note illustrates the approximate exponential disappearance of pharmacologic response for several drugs and indicates how this knowledge may be applied pharmaceutically.

EXAMPLES FROM THE LITERATURE

Pyretogenic Effects.—Gogerty and Dille (9) measured the pyretogenic effect of *d*-lysergic acid diethylamide (LSD) and of *d*-lysergic acid morpholide (LSM) following i. v. administration into rabbits. The disappearance of fever appeared to be approximately exponential when we replotted their data for 50 mcg./Kg. doses on semilog paper (Fig. 1). By graphical estimation, the half-lives ($t_{\frac{1}{2}}$) for LSM and LSD were approximately 80 and 430 min., and the specific velocity constants ($k = 0.693/t_{\frac{1}{2}}$) 0.0087 $^{-min.}$ and 0.0016 $^{-min.}$, respectively. It is of interest that the replacement of the diethylamine side chain by the morpholine moiety results in a more rapid elimination of the compound from its receptors. This may also serve to explain the lower peak activity of LSM, although one cannot deny possible differences in affinity and intrinsic activity between LSM and LSD.

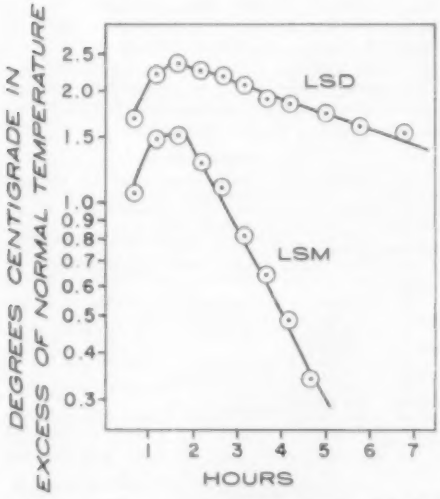

Fig. 1.—Effect on rabbit rectal temperature after intravenous administration of 50 mcg./Kg. of LSM and LSD, respectively. Each curve represents mean values of six rabbits. Data for plots from Gogerty and Dille (9).

Antisialagogue Effects.—Carter, *et al.* (10), obtained some limited human data on salivary response following the oral administration of tincture of belladonna. Again, if the data are replotted on semilog paper, the disappearance of the antisialagogue activity appears to follow an exponential course (Fig. 2). The half-life is approximately 1 to 1.5 hours and the velocity constant about 0.6 $^{-hr.}$.

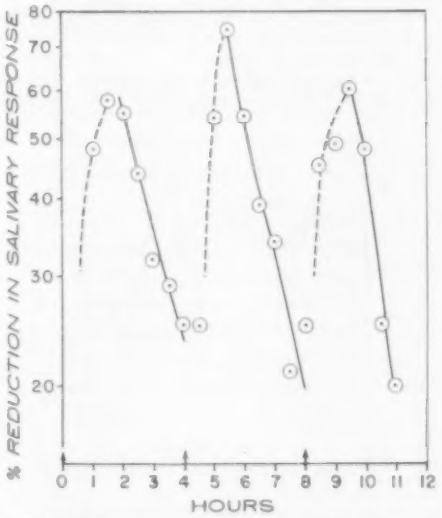

Fig. 2.—Mean response of four experiments in two human subjects given 2.4 ml. tincture of belladonna at zero, four, and eight hours. Data for plots from Carter, *et al.* (10).

EXPERIMENTAL

Twelve male Dierolf mice were given 5 mg./Kg. oral doses of the new anticholinergic agent SKF 5515-I (9-methyl-3-oxo-9-azabicyclo-[3,3,1]-nonan-7-yl-benzilate maleate) (11). At the next five hourly intervals, additional maintenance doses of 1 mg./Kg. orally were given. Measurements of mydriasis were made under constant illumination using an ocular micrometer; these readings were recorded half-hourly in terms of arbitrary units (1 unit ≅ 0.2 mm.). The size of the maintenance dose was calculated on the basis of previous experiments (12), in which SKF 5515-I displayed an apparent mydriatic half-life of 2.0 hours in mice; the corresponding velocity constant was 0.35 $^{-hr.}$. Thus, letting W = amount of drug in the body which is

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The authors wish to acknowledge the technical assistance of Mr. Gordon Black.

activating the mydriatic receptors at a desired level (mg./Kg.), W_0 = extrapolated "initial" amount of drug at $t = 0$, which approximates dose if absorption is rapid (mg./Kg.), t = time (hours), and k = constant for removal of drug from receptors (hours^{-1})

$$W = W_0 e^{-kt} \quad \text{(Eq. 1)}$$

$$W_0 - W = W_0(1 - e^{-kt}) \quad \text{(Eq. 2)}$$

To maintain the intensity of the one-hour mydriasis with hourly sustaining doses, we may substitute in the last equation $W_0 = 5$, $t = 1$, and $k = 0.35$, whereupon $W_0 - W = 1.5$ mg./Kg. and $W = 3.5$ mg./Kg. The replenishment dose is then about $3.5(1 - e^{-0.35}) = 1.0$ mg./Kg. hourly.

RESULTS AND DISCUSSION

In Fig. 3 it is observed that a fairly constant level of mydriasis was maintained by the replenishment doses of SKF 5515-I. After the last dose, the $t_{\frac{1}{2}}$ for disappearance of mydriasis was 1.3 hours, which in turn compares favorably with the value of 2 hours determined previously.

In utilizing the preceding equations and calculations, no attempt was made to interpret mechanisms of drug action. Furthermore, the assumption of first-order disappearance of pharmacologic response may be only approximately correct. As has been shown by Hill and others (4, 13, 14), the drug receptor reaction might be theoretically and experimentally described by a reversible second-order reaction. However, first-order rate equations are more simply handled than those of a higher order. It becomes a practical expedient to interpret data in terms of pseudo first-order reaction rate kinetics when possible.

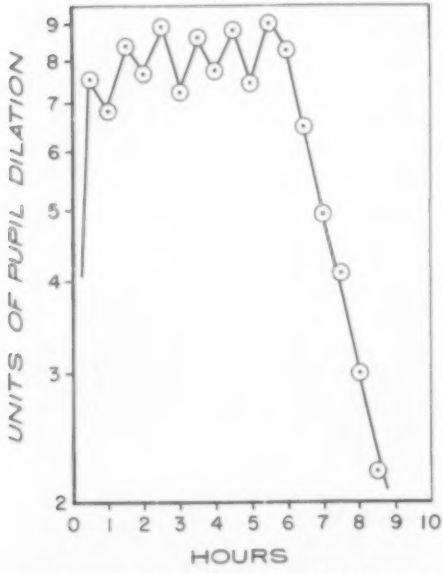

Fig. 3.—Mean pupillary dilation in 12 mice receiving 5 mg./Kg. of SKF 5515-I orally at zero time followed by 1 mg./Kg. at one, two, three, four, and five hours. Each unit of pupil dilation equals approximately 0.2 mm.

The utility of the first-order rate kinetic constants for the design of oral sustained action products, for example, may be illustrated. For a sustained mydriatic action at a 3.5 mg./Kg. level of SKF 5515-I, where absorption from the sustained release portion of the dose is zero order, where $k = 0.35$, and where the rate of drug elimination, dE/dt, should ideally equal the rate of absorption, dA/dt, i. e., where $dW/dt = (dA/dt) - (dE/dt) = 0$, one may compute that the constant rate of absorption should be $dA/dt = kW = 0.35 \times 3.5 = 1.2$ mg./Kg./hour. This is very nearly equal to the replenishment dose required when administered every hour as illustrated in previous calculations.

If, for example, sustained pupil dilation at this level is required for about four hours, the equation for estimating the total dose, initial release, and the maintenance portion of the sustained action dosage unit may be written

$$\underset{\text{(total dose)}}{Wt} = \underset{\text{(initial release)}}{W_0} + \underset{\text{(maintenance portion)}}{\int_{1\text{ hr.}}^{4\text{ hr.}} kW\,dt} \quad \text{(Eq. 3)}$$

$$= W_0 + (k \times W \times 3)$$
$$= 5 + (0.35 \times 3.5 \times 3)$$
$$= 8.6 \text{ mg./Kg.}$$

The dosage unit, therefore, should contain 5 mg./Kg. for initial release, with 3.6 mg./Kg. available for relatively uniform absorption over about the one to four hour interval. The equation and calculation may assume minor variations depending upon the rate of drug absorption, accuracy with which W may be determined, and other assumptions.

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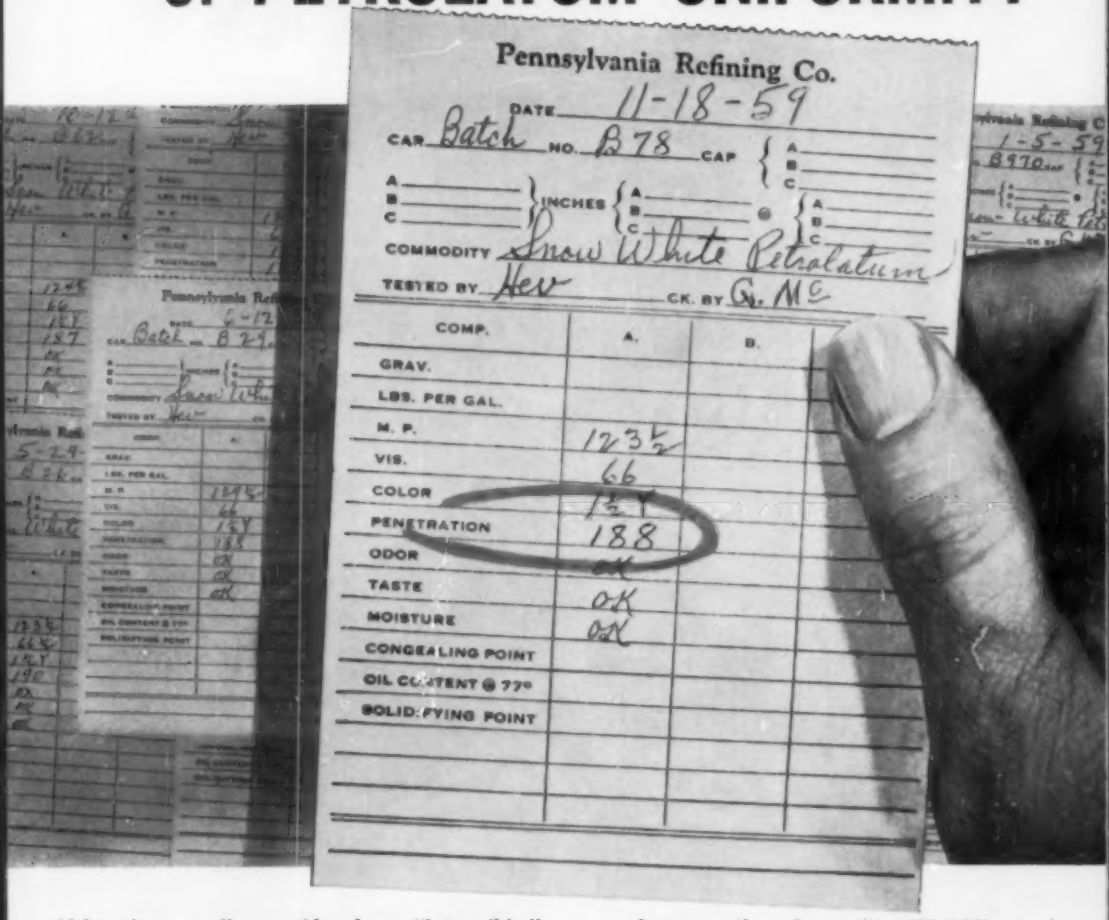

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